Enclosures - letter from Dr. Gerald McEwen, CTFA, on June 8, 2005 in response to request for additional information/public comments on Toxicological Program (70 Federal Register 23877): Imidazolidinyl urea:

Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test). Test Article AT0214 (Germall II - Diazolidinyl Urea). Microbiological Associates Study No. T2039.501. 42 pages.

Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test). Test Article AT0214, Lot No. GT115. Diazolidinyl Urea (Germall II) RI 1157. Microbiological Associates Study No. T2039.501008. 35 pages.

Germall II. Mouse Micronucleus Test for Chromosomal Aberrations. PH 309-SU-001-86. 67 pages.

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

TEST ARTICLE AT0214
LOT NO. GT115

Diazolidinyl Urea (Germall II) RI 1157

MICROBIOLOGICAL ASSOCIATES

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Whittaker

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Sponsor:

Testing Facility: 1530 East Jefferson Street
Rockville, Maryland 20852

Study No.: T2039.501008

Test Article I.D.: AT0214

Test Article Lot No.: GT115

Test Article Description: White Powder

Storage Conditions: Room Temperature with Desiccation; Protected from Light

Date Received: 6/7/83

Date Study Started: 8/11/83

Date Study Completed: 9/15/83

Report Date: 9/15/83

Study Coordinator:

Study Director: Steve R. Haworth, Ph.D.
Microbiological Associates

Steve R. Haworth 9/15/83
Steve R. Haworth, Ph.D. Date
Study Director

Timothy E Lawlor 9.15.83
Timothy E. Lawlor Date
Alternate Study Director

Jeanene K Burke 9/15/83
Jeanene K. Burke Date
Group Leader

Sheila M Olewine 9/15/83
Sheila M. Olewine Date
Biologist

Robin J. Plunkett 9/15/83
Robin J. Plunkett Date
Biologist

Linda M. Coyle 9/15/83
Linda M. Coyle Date
Biologist

QUALITY ASSURANCE STATEMENT

Study Title: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

Study Number: T2039.501008

Study Director: S. Haworth, Ph.D.

Initiation Date: August 11, 1983

Review Completed Date: September 15, 1983

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc. are examined in order to assure that the study is performed in accordance with the Good Laboratory Practices regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
8/11/83	Protocol review	8/11/83	8/11/83
8/17/83	Preparation of S-9 mixes	8/17/83	8/18/83
9/15/83	Final report	9/15/83	9/15/83

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nona S. Karten 9/15/83

Nona S. Karten Date
Associate Director RA/QA

Introduction

test article AT0214, Lot No. GT115 was originally received on June 7, 1983, for testing in the Salmonella/mammalian-microsome mutagenicity assay (Ames test) using five tester strains, TA98, TA100, TA1535, TA1537 and TA1538, both with and without activation by Aroclor induced rat liver microsomes.

In this original Ames study, the test article was shown to cause slight increases in TA98 and TA1537 revertants per plate in the presence of 10% rat S-9 homogenate per ml of S-9 mix. Since the increase in revertants appeared to be S-9 dependent, a study was designed to investigate 1) the effect of increasing and decreasing the S-9 concentrations on revertant recovery, and 2) the effect of S-9 prepared from the livers of Aroclor 1254 induced hamsters compared to S-9 prepared from the livers of Aroclor 1254 induced rats.

The study employed tester strains TA98 and TA1537 and the following concentrations of S-9 homogenate per ml of S-9 mix for both hamster and rat S-9 preparations:

5% S-9 homogenate = .05 ml S-9 homogenate/ml of S-9 mix
10% S-9 homogenate = .10 ml S-9 homogenate/ml of S-9 mix
30% S-9 homogenate = .30 ml S-9 homogenate/ml of S-9 mix

Conclusions

The results of the specially designed Salmonella/mammalian-microsome mutagenicity assay indicate that under the conditions of this study, test article AT0214, Lot No. GT115 did not cause a positive response on either of the tester strains under any of the experimental conditions investigated.

The increases in TA98 and TA1537 revertants per plate were similar to those obtained in previous studies. Additionally, neither the species from which the S-9 was derived nor the

concentration of the homogenate used in the S-9 mix appeared to greatly influence the magnitude of the observed revertant increases.

MATERIALS AND METHODS[1]

Media Preparation

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 8 g/liter Difco Bacto Agar and 5 g/liter NaCl. After autoclaving, the molten agar was distributed in 100 ml aliquots into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 ml/100 ml agar of a sterile solution which contained 0.5 mM L-histidine and 0.5 mM D-biotin. Twenty-five ml of sterile deionized water was added per 100 ml top agar when it was used in assays without metabolic activation. This ensured that final top agar and amino acid supplement concentrations were the same on plates with or without metabolic activation.

Top Agar for Viable Count Determination: Minimal top agar as described above was supplemented with 35 ml/100 ml agar of a sterile solution which contained 1.4 mM L-histidine and 0.12 mM D-biotin.

Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E.[2]

Nutrient Broth: Nutrient broth used for growing overnight cultures of the tester strains contained 25 g per liter of Nutrient Broth No. 2 (Oxoid).

Nutrient Bottom Agar: Nutrient bottom agar was Vogel-Bonner[3] minimal medium E supplemented with 25 g per liter of Nutrient Broth No. 2 (Oxoid).

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[1]The experimental materials, methods and procedures are based on those described by Ames, B. N., et al. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31: 347-364, 1975.

[2]Vogel, H. J. and D. M. Bonner, Acetylornithinase of E. coli: partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).

[3]Ibid.

Tester Strain Diluent: Diluent for tester strain titering contained Vogel-Bonner salt solution[4] supplemented with 10% Nutrient Broth.

Test Article Diluent: The solvent used for diluting the test article was deionized, distilled H_2O.

Tester Strains

The tester strains used were the histidine auxotrophs TA98 and TA1537 described by Ames[5].

GENOTYPE OF THE TA STRAINS USED FOR MUTAGEN TESTING

Histidine mutation		Additional mutations		
hisC3076	hisD3052	LPS	Repair	R factor
	TA98	rfa	uvrB	+R
TA1537		rfa	uvrB	–

The tester strains possess characteristics which greatly enhance their sensitivity to mutagenic materials.

Both strains possess the rfa wall mutation which has resulted in the loss of much of the lipopolysaccharide layer that coats the surface of the bacteria. This allows the entry into the bacterial cells of large ring compounds that would otherwise be excluded by a normal intact cell wall. Secondly, a stable mutation resulting in the loss of an excision repair system (uvrB) further enhances both tester strains' sensitivity to some mutagens. Finally, strain TA98 contains the pkM101 plasmid which further increases the sensitivity of this strain to some mutagens.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frame shift mutagens.

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[4]Vogel, H. J., et al., op cit.

[5]Ames, B. N., et al., op cit.

Tester strains in use at Microbiological Associates were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Tester strain stocks were stored in liquid nitrogen, and fresh cultures were inoculated directly from these frozen stocks. Broth cultures were grown overnight at 37°C with shaking. At the time of its use in the mutagenicity assay, each culture was checked, as described by Ames, for the presence of the rfa wall mutation and strain TA98 was checked for the presence of the pkM101 plasmid.[6]

Plating Procedures for the Mutagenicity Assay

Test System Identification: Each plate was labeled using indelible ink with a code system which identifies the test article, test phase, dose level and activation as described in detail in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

Test Article: The test article was solubilized and serially diluted immediately before its use in the mutagenicity assay. Five doses of the test article were plated with TA98 and TA1537 with metabolic activation. All positive controls, solvent controls and test article doses were plated in triplicate. With metabolic activation, 50 µl of tester strain, 50 µl of solvent or test article, and 0.5 ml of the appropriate S-9 mix were added to 2.0 ml of molten selective top agar at 45°C. After vortexing, the mixture was overlaid onto the surface of 25 ml of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37°C.

Positive Controls: All combinations of positive controls and tester strains plated along with the assay are listed below:

Strain	Activation	Positive Controls	Conc. per Plate
TA98	+	2-Aminoanthracene	4.0 µg
TA98	-	2-Nitrofluorene	5.0 µg
TA1537	+	2-Aminoanthracene	4.0 µg
TA1537	-	9-Aminoacridine	75 µg

[6]Ames, B. N., et al., op cit.

Source and Grade

9-Aminoacridine (CAS #90-45-9), Sigma Chemical Co., grade II, ~90% pure

2-Aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade

2-Nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98% pure

Tester Strain Titers: Tester strain titers were determined by viable count assays on nutrient agar plates. The averaged number of cells plated per plate are reported on the individual strain data forms.

Test Article Sterility Determination: The most concentrated test article dilution for the mutagenicity assay was checked for sterility by plating a 50 µl aliquot of the dilution on selective agar.

Liver Microsomal Enzymes

Preparation of S-9 Homogenate: Rat liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 at 500 mg/kg. Hamster liver microsomal enzymes were prepared from male Syrian hamsters that had been injected with Aroclor 1254 at 500 mg/kg. The Aroclor was diluted in corn oil to a concentration of 200 mg/ml. Five days after their i.p. injection with the Aroclor, the animals were sacrificed by decapitation, and their livers were excised.

The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 0-4°C. The excised livers were placed in approximately 20 ml of 0.15M KCl contained in a pre-weighed beaker. After weighing the liver, it was transferred to another beaker containing 3 volumes of 0.15M KCl (3 ml/g of wet liver) where it was minced with sterile scissors. The minced liver was homogenized and centrifuged at 9000 x g for 10 minutes. The supernatant (referred to by Ames as the S-9 fraction)

was decanted, and small aliquots were distributed into freezing ampules which were stored at $\leq -70^{\circ}C$.

Preparation of S-9 Mix: The S-9 mixes were prepared immediately before their use in the mutagenicity assay.

For both rat and hamster activation, three different S-9 mixes were used in this study. The mixes differ only in the amount of S-9 homogenate added per ml of S-9 mix. The components per ml of each S-9 mix are indicated below:

5% S-9 Homogenate

H_2O	0.61 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.05M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.05 ml
	1.00 ml

10% S-9 Homogenate

H_2O	0.56 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.05M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.10 ml
	1.00 ml

30% S-9 Homogenate

H_2O	0.36 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.05M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.30 ml
	1.00 ml

Each plate received 0.5 ml of the S-9 mix.

Colony Counting

Revertant colonies for a given tester strain within a given test article dilution series were counted either entirely by automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

The condition of the background bacterial lawn was evaluated for evidence of test article toxicity, by using a dissecting microscope. This toxicity was scored relative to the solvent control plate and recorded along with the revertant count for that plate on the individual strain data forms using the code system on page 19.

Analysis of Data

All platings were done in triplicate. For each triplicate plating, an average and standard deviation were calculated. The calculations were done on a Hewlett-Packard HP-25 programmable calculator which employs the following equations:

Average ($\bar{x}$)

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

Standard Deviation (S_x)

$$S_x = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

Evaluation of Mutagenicity Assay Data

For a test article to be considered positive, it must cause at least a doubling in the mean revertants per plate of at least one tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response

to increasing concentrations of the test article. In those cases where the observed dose-responsive increase in TA1537 revertants per plate is less than three-fold, the response must be reproducible.

Archives

All experimental records of the study are maintained in the Microbiological Associates' archives located at 1530 East Jefferson Street, Rockville, Maryland, 20852.

Nona Karten, of the Regulatory Affairs and Quality Assurance Unit, is responsible for maintaining the archives.

Stability of the Test Article

The stability of the test article under the actual experimental conditions used in this study was not determined by Microbiological Associates.

RESULTS

SALMONELLA MUTAGENICITY ASSAY

TABLE 1

T2039.501008 — Study Number

AT0214, Lot No. GT115 — Test Article Identification

T2039-B6 — Experiment Number

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Rat 5%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
	Concentration (µg per plate)								
*					2	3			
Revertants per plate	32	23	35	46	40	21			
					2	3			
	39	17	34	34	45	23			
					2	2			
	27	28	39	33	42	57			
Averaged Revertants	33	23	36	38	42	34			
Standard Deviation	6	6	3	7	3	20			

T2039-B6 — Experiment Number

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Hamster 5%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
	Concentration (µg per plate)								
*			5		2	3			
Revertants per plate	26	31	0**	28	25	23			
			5	5	2	3			
	26	33	0**	0**	39	25			
			5	5	2	3			
	31	27	0**	0**	45	33			
Averaged Revertants	28	30	-	28	36	27			
Standard Deviation	3	3	-	-	10	5			

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12/17/82

*Background bacterial lawn evaluation code
**The absence of revertants and background lawn colonies on these plates at non-toxic dose levels indicates a probable plating procedural error. For this report, these counts have not been used in the average or standard deviation calculations.

SALMONELLA MUTAGENICITY ASSAY

TABLE 2

T2039,501008 (Study Number) — AT0214, Lot No. GT115 (Test Article Identification)

T2039-B6 (Experiment Number)

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Rat 10%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
	Concentration (µg per plate)								
*					2	3			
Revertants per plate	22	33	20	37	40	42			
	28	32	23	35	48	34			
	28	21	28	50	43	33			
Averaged Revertants	26	29	24	41	44	36			
Standard Deviation	3	7	4	8	4	5			

T2039-B6 (Experiment Number)

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Hamster 10%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
	Concentration (µg per plate)								
*					2	3			
Revertants per plate	34	39	36	47	49	30			
	39	24	32	46	53	40			
	33	30	36	44	53	37			
Averaged Revertants	35	31	35	46	52	36			
Standard Deviation	3	8	2	2	2	5			

Form No. MA-160
12/17/82

*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

TABLE 3

T2039.501008 — Study Number

AT0214, Lot No. GT115 — Test Article Identification

T2039-B6 — Experiment Number

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Rat 30%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
*					2	2			
Revertants per plate	27	31	28	47	43	41			
					2	2			
	31	25	30	35	51	29			
					2	2			
	30	30	33	31	32	39			
Averaged Revertants	29	29	30	38	42	38			
Standard Deviation	2	3	3	8	10	6			

Concentration (µg per plate)

T2039-B6 — Experiment Number

Strain: TA98
Date Plated: 8/17/83
Cells Seeded: 1.4 x10^8
Liver Microsomes: Hamster 30%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	100	200	400	500	600			
*						2			
Revertants per plate	23	25	23	34	53	46			
						2			
	31	30	31	37	53	52			
						2			
	37	35	22	39	47	70			
Averaged Revertants	30	30	25	37	51	56			
Standard Deviation	7	5	5	3	3	12			

Concentration (µg per plate)

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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

TABLE 4

T2039.501008 (Study Number) — AT0214, Lot No. GT115 (Test Article Identification)

T2039-B6 (Experiment Number)

		Solvent Control H_2O 50 µl	100	200	400	500	600			
Strain: TA1537	*				2	2	3			
Date Plated: 8/17/83	Revertants per plate	9	5	9	12	9	10			
Cells Seeded: 1.5 x10^8		8	7	5	8	13	6			
Liver Microsomes: Rat 5%		12	8	9	11	12	8			
Colonies Counted by:	Averaged Revertants	10	7	8	10	11	8			
Hand ☒	Standard Deviation	2	2	2	2	2	2			
Machine ☐										

T2039-B6 (Experiment Number)

		Solvent Control H_2O 50 µl	100	200	400	500	600			
Strain: TA1537	*				2	2	3			
Date Plated: 8/17/83	Revertants per plate	7	9	10	10	10	6			
Cells Seeded: 1.5 x10^8		12	8	6	12	9	5			
Liver Microsomes: Hamster 5%		8	5	9	10	8	4			
Colonies Counted by:	Averaged Revertants	9	7	8	11	9	5			
Hand ☒	Standard Deviation	3	2	2	1	1	1			
Machine ☐										

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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

TABLE 5

T2039.501008 — Study Number

AT0214, Lot No. GT115 — Test Article Identification

T2039-B6 — Experiment Number

Strain: TA1537
Date Plated: 8/17/83
Cells Seeded: 1.5 x10^8
Liver Microsomes: Rat 10%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 100	200	400	500	600			
*				2	2	3			
Revertants per plate	7	3	7	10	8	4			
				2	2	3			
	12	6	11	14	11	8			
				2	2	2			
	6	7	9	7	12	12			
Averaged Revertants	8	5	9	10	10	8			
Standard Deviation	3	2	2	4	2	4			

T2039-B6 — Experiment Number

Strain: TA1537
Date Plated: 8/17/83
Cells Seeded: 1.5 x10^8
Liver Microsomes: Hamster 10%
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 100	200	400	500	600			
*				2	2	2			
Revertants per plate	7	8	8	10	16	11			
				2	2	2			
	4	2	12	11	7	12			
				2	2	2			
	6	5	6	9	6	7			
Averaged Revertants	6	5	9	10	10	10			
Standard Deviation	2	3	3	1	6	3			

Form No. MA-160
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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

TABLE 6

T2039.501008 (Study Number) — AT0214, Lot No. GT115 (Test Article Identification)

T2039-B6 (Experiment Number)

		Solvent Control H_2O 50 µl	100	200	400	500	600			
		Concentration (µg per plate)								
Strain: TA1537	*				2	2	2			
Date Plated: 8/17/83	Revertants per plate	18	9	7	9	22	12			
Cells Seeded: 1.5 x10^8		9	7	17	9	2 14	3 9			
Liver Microsomes: Rat 30%		13	7	14	8	2 15	3 18			
Colonies Counted by:	Averaged Revertants	13	8	13	9	17	13			
Hand ☒	Standard Deviation	5	1	5	1	4	5			
Machine ☐										

T2039-B6 (Experiment Number)

		Solvent Control H_2O 50 µl	100	200	400	500	600			
		Concentration (µg per plate)								
Strain: TA1537	*					2	2			
Date Plated: 8/17/83	Revertants per plate	5	7	9	11	7	12			
Cells Seeded: 1.5 x10^8		14	15	8	6	2 9	2 10			
Liver Microsomes: Hamster 30%		6	5	6	9	10	2 11			
Colonies Counted by:	Averaged Revertants	8	9	8	9	9	11			
Hand ☒	Standard Deviation	5	5	2	3	2	1			
Machine ☐										

Form No. MA-160
12/17/82

*Background bacterial lawn evaluation code

SALMONELLA MUTAGENESIS ASSAY

Positive Controls

Table 7

T2039.501008 Study Number	T2039-B6 Experiment Number	AT0214, Lot No. GT115 Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
8/17/83	TA98	2-Nitrofluorene	5.0 µg	None	1166	1124	1227	1172	52
8/17/83	TA98	2-Aminoanthracene	4.0 µg	Rat 5%	3319	3416	3312	3349	58
8/17/83	TA98	2-Aminoanthracene	4.0 µg	Rat 10%	2039	1914	2092	2015	91
8/17/83	TA98	2-Aminoanthracene	4.0 µg	Rat 30%	795	915	780	830	74
8/17/83	TA98	2-Aminoanthracene	1.5 µg	Hamster 5%	2982	2899	3203	3028	157
8/17/83	TA98	2-Aminoanthracene	1.5 µg	Hamster 10%	2593	2725	2489	2602	118
8/17/83	TA98	2-Aminoanthracene	1.5 µg	Hamster 30%	506	453	554	504	51

Colonies were machine counted.

SALMONELLA MUTAGENESIS ASSAY

Positive Controls

Table 8

T2039.501008 Study Number	T2039-B6 Experiment Number	AT0214, Lot No. GT115 Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
8/17/83	TA1537	9-Aminoacridine	75 µg	None	438	333	426	399	57
8/17/83	TA1537	2-Aminoanthracene	4.0 µg	Rat 5%	500	552	457	503	48
8/17/83	TA1537	2-Aminoanthracene	4.0 µg	Rat 10%	297	295	287	293	5
8/17/83	TA1537	2-Aminoanthracene	4.0 µg	Rat 30%	65	99	66	77	19
8/17/83	TA1537	2-Aminoanthracene	1.5 µg	Hamster 5%	410	455	444	436	23
8/17/83	TA1537	2-Aminoanthracene	1.5 µg	Hamster 10%	296	331	316	314	18
8/17/83	TA1537	2-Aminoanthracene	1.5 µg	Hamster 30%	58	62	58	59	2

Colonies were machine counted.

Form No. MA-161
1/7/83

BACTERIAL BACKGROUND LAWN EVALUATION CODES

The condition of the background bacterial lawn is evaluated, first macroscopically and then microscopically (using a dissecting microscope). The evaluation is recorded using the following code:

Code	Definition	Characteristics
1 or blank	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
5	Absent	Distinguished by a complete lack of any microcolony background lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Distinguished by noticeable precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
HP	Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Thus, 3-MP would indicate a plate observed to have a moderately reduced background lawn with a marked amount of precipitate which required a hand count.

APPENDIX

PROTOCOL AMENDMENT

Date: September 15, 1983

Sponsor:

Sponsor's Test Article Designation: AT0214

Study No.: T2039.501008

Protocol No.: SPGT501008 080983

Protocol Title: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

1. Section 6.3.2, S-9 Mix; the stock solution listed as "0.04M G-6-P" should be "0.05M G-6-P".

Reason for Amendment:

Typographical error in protocol preparation.

APPROVAL:

Steve R. Haworth 9/15/83
Steve R. Haworth, Ph.D. Date
Study Director

9/16/83
Ph.D. Date
Study Coordinator

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article (or its metabolites) based on its ability to induce back mutations at selected loci of selected strain(s) of Salmonella typhimurium in the presence of exogenous metabolic activation by three different concentrations of induced rat and hamster liver microsomes.

2.0 TEST ARTICLE

2.1 Identification: AT0214

2.2 Analysis:
The sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability of the dosing solutions.

3.0 SPONSOR

3.1 Name:

3.2 Address:

3.3 Authorized Representative:

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Steve R. Haworth, Ph.D.

5.0 TEST SYSTEM

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a wide range of chemical classes.

The Salmonella typhimurium histidine auxotroph tester strains to be used may include TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames (Ames, et al., Mutation Research 31:347-364, 1975). The actual strains to be used will be as follows:

TA98, TA1537 — SRH (Initials) 8/10/83 (Date)

GENOTYPE OF THE TA STRAINS USED FOR MUTAGEN TESTING

Histidine mutation			Additional mutations		
hisG46	hisC3076	hisD3052	LPS	Repair	R factor
TA1535	TA1537	TA1538	rfa	uvrB	-
TA100		TA98	rfa	uvrB	+R

All of the tester strains contain, in addition to a mutation in the histidine operon, two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded by a normal intact cell wall.

The second mutation is a deletion in the uvrB gene which results in a deficient DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Finally, strains TA98 and TA100 also contain the pkM101 plasmid (carrying the R-factor) which further increases the sensitivity of these two strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be due to its coding for an error-prone DNA repair polymerase.

TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frame shift mutagens. TA100 and TA1535 are reverted by mutagens that cause base substitutions.

5.1 Source
Tester strains in use at Microbiological Associates were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

5.2 Storage
All Frozen Permanent and Working Stocks of the tester strains will be stored in liquid nitrogen. Working Stocks will be prepared by growing a fresh overnight culture inoculated by a scrape of the Frozen Permanent Stock, adding DMSO (.09 ml/ml of culture) and freezing away small aliquots (0.1 - 0.2 ml) in glass vials.

5.3 Overnight Culture Preparation
Overnight cultures will be prepared by removing a Working Stock vial from the liquid nitrogen freezer and allowing it to thaw. A loopful of the thawed aliquot will be transferred to a baffled shake flask containing approximately 50 ml of culture media. The inoculated flask will be placed in a shaker/incubator at $37^{\circ}C$.

5.4 Harvesting of Cultures
All cultures will be harvested by monitoring optical density rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately $1\text{-}2 \times 10^9$ cells per ml.

5.5 Genotype Characterization
On the day of their use in the mutagenicity assay, all tester strain cultures will be checked for the following genetic markers:

5.5.1 The presence of the rfa wall mutation will be confirmed by demonstration of sensitivity to crystal violet.

5.5.2 The presence of the pkM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstration of resistance to Ampicillin.

5.5.3 Spontaneous reversion frequencies that are characteristic of the respective strains will be demonstrated by plating aliquots of the culture on selective media.

6.0 EXPERIMENTAL DESIGN

The test article will be tested at a minimum of five dose levels along with appropriate solvent and positive controls on Salmonella tester strains which may include TA98, TA100, TA1535, TA1537 and TA1538 with and without metabolic activation. Following an approximate 48 hour incubation at 37°C, revertant colonies per plate will be counted.

6.1 Dose Levels

Unless other supporting data is available, a preliminary toxicity study will be conducted. Using TA100 as the indicator strain, each test article will be checked for toxicity up to a concentration of 10 mg/plate if solubility/miscibility permits. Test articles which exhibit limited solubility/miscibility will be tested for toxicity up to the maximum workable concentration attainable in the solvent of choice. The toxicity determination will be conducted in the presence of exogenous metabolic activation. An aliquot from each of at least eight dilutions of the test article will be plated with an overnight TA100 culture on selective minimal agar. Toxicity is detectable as a decrease in the number of revertant colonies occurring per plate and/or by a thinning or disappearance of the background bacterial lawn. The highest concentration of test article used in the subsequent mutagenicity assay will be that which gives a detectable reduction of revertants on the selective plates and/or a thinning or disappearance of the background bacterial lawn.

If no toxicity is observed, then the highest dose level used in the mutagenicity assay will be 10 mg/plate unless:

1) The test article exhibits limited solubility or is not uniformly dispersible in the solvent of choice.

2) The test article precipitates heavily in the top agar.

3) There is insufficient test article available to either demonstrate toxicity or achieve a maximum dose level of 10 mg/plate.

4) The study coordinator indicates an alternate top dose level.

6.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally

described by Ames (Ames, et al., Mutation Research 31:347-364, 1975). This methodology has been shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, solvent controls and positive controls will be plated in triplicate.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Homogenate Preparation
The preparation of the microsomal enzyme fraction will be carried out with sterile glassware and solutions at 0-4°C. Excised livers will be placed in approximately 20 ml of 0.15M KCl contained in a pre-weighed beaker. After the liver is weighed, it will be transferred to another beaker containing 3 volumes of 0.15M KCl (3 ml/g of wet liver) where it will be minced with sterile scissors. The minced liver will be homogenized and centrifuged at 9000 x g for 10 minutes. The supernatant (referred to by Ames as the S-9 fraction) will be decanted, and small aliquots will be distributed into freezing ampules which will be stored at $\leq$ -70°C.

6.3.1.2 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by deSerres (deSerres, et al., Science 203:563-565, 1979).

6.3.1.3 Species, Strain, Sex, Inducer
Liver microsomal enzymes will be prepared from male Sprague-Dawley rats and male Syrian hamsters that have been injected with Aroclor 1254 at 500 mg/kg. The Aroclor will be diluted in corn oil to a concentration of 200 mg/ml. Five days after i.p. injection with the Aroclor, the rats and hamsters will be sacrificed by decapitation, and their livers will be excised.

6.3.2 S-9 Mix

The S-9 mixes will be prepared immediately prior to their use in any experimental procedure.

Three different S-9 mixes will be used in this study. The mixes will differ only in the amount of S-9 homogenate added per ml of S-9 mix. The components per ml of each S-9 mix are indicated below:

5% S-9 Homogenate

H_2O	0.61 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.04M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.05 ml
	1.00 ml

10% S-9 Homogenate

H_2O	0.56 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.04M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.10 ml
	1.00 ml

30% S-9 Homogenate

H_2O	0.36 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.04M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.30 ml
	1.00 ml

Each plate will receive 0.5 ml of the S-9 mix.

6.4 Controls

6.4.1 Positive Controls
All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Strain	Activation	Positive Controls	Conc. per Plate
TA98	+	2-aminoanthracene	4.0 ug
TA98	-	2-nitrofluorene	5.0 ug
TA100	+	2-aminoanthracene	4.0 ug
TA100	-	sodium azide	5.0 ug
TA1535	+	2-aminoanthracene	4.0 ug
TA1535	-	sodium azide	5.0 ug
TA1537	+	2-aminoanthracene	4.0 ug
TA1537	-	9-aminoacridine	75 ug
TA1538	+	2-aminoanthracene	4.0 ug
TA1538	-	2-nitrofluorene	5.0 ug

6.4.2 Solvent Controls
Appropriate solvent controls will be plated for all strains with exogenous metabolic activation. Solvents compatible with this test system in order of preference include but will not be limited to: Deionized distilled H_2O, dimethylsulfoxide (CAS #67-68-5), acetone (CAS #67-64-1), and ethanol (CAS #64-17-5).

6.4.3 Sterility Controls

6.4.3.1 The most concentrated test article dilution will be checked for sterility.

6.4.3.2 The S-9 mix will be checked for sterility.

6.4.4 Tester Strain Titers
Each tester strain titer will be determined by plating an appropriate dilution of each overnight culture on complete agar.

7.0 METHODS

7.1 Plating Procedures for the Mutagenicity Assay
The test article will be solubilized and serially diluted immediately before its use in the mutagenicity assay. S-9 mixes will also be prepared immediately prior to their use in the mutagenicity assay.

At least five doses of the test article will be plated with the appropriate tester strains, with each exogenous metabolic activation system.

Fifty ul of tester strain, 50 ul of solvent or test article solution and 0.5 ml of S-9 mix will be added to 2.0 ml of molten selective top agar at 45°C. After vortexing, the mixture will be overlayed onto the surface of 25 ml of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 hours at 37°C. When necessary, aliquots of other than 50 ul of test article/solvent will be plated.

7.2 Test System Identification
Each plate will be labeled using indelible ink with a code system which identifies the test article, test phase, dose level, strain and activation type as described in detail in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.3 Colony Counting
Revertant colonies for a given tester strain within a given test article dilution series will be counted either entirely by automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting will be counted manually.

7.3.1 Background Bacterial Lawn Evaluation
The condition of the background bacterial lawn on plates in the assay will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the solvent control plate and recorded along with the revertant count for that plate.

7.4 Analysis of Data
For all replicate platings, the mean revertants per plate and the standard deviation will be calculated.

8.0 EVALUATION OF TEST RESULTS

For a test article to be considered positive, it must cause at least a doubling in the mean revertants per plate of at least one tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article. In those cases where the observed dose-responsive increase in TA1537 or TA1538 revertants per plate is less than three-fold, the response must be reproducible.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the assay to be considered valid:

9.1 Tester Strain Integrity

9.1.1 rfa Wall Mutation
In order to demonstrate the presence of the deep rough wall mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

9.1.2 pkM101 Plasmid R-factor
In order to demonstrate the presence of the pkM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to Ampicillin.

9.1.3 Characteristic Number of Spontaneous Revertants
All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate. The acceptable ranges are as follows:

TA98	10 - 50
TA100	80 - 240
TA1535	5 - 45
TA1537	3 - 21
TA1538	5 - 35

9.1.4 Tester Strain Titers
In order to ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than 1×10^9 but less than 4×10^9.

9.1.5 Positive Control Values
Positive control values must exhibit at least a three fold increase in the number of revertants per plate over the average value for the solvent control for the respective strain.

9.2 Toxicity

9.2.1 Minimum Number of Dose Levels
A minimum of three non-toxic dose levels are required to evaluate assay data.

10.0 FINAL REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of data.

Results of the preliminary toxicity determinations will be presented which will include the number of revertants per plate and a background bacterial lawn evaluation for each dose level.

Results presented for the mutagenicity assay will include the number of revertants per plate with a corresponding background bacterial lawn evaluation, along with a mean and standard deviation for all replicate platings.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A sample of the Test Article will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? _____
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned? _____

13.0 SCHEDULE OF EVENTS

13.1 Proposed Initiation Date: 8/11/83

13.2 Scheduled Completion Date: 8/31/83

14.0 REFERENCES

Ames, B.N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/Mammalian-Microsome Mutagenicity test. Mutation Research 31:347-364, 1975.

deSerres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

8/9/83
DATE PROTOCOL APPROVED BY SPONSOR

Steve R. Haworth 8/11/83
STUDY DIRECTOR DATE

Securing the Industry's Future Since 1894

CTFA
Cosmetic, Toiletry, and Fragrance Association

Pamela G. Bailey
President & CEO

June 2, 2005

Dr. Scott A. Masten
Office of Chemical Nomination and Selection
NIEHS/NTP
111 T.W. Alexander Drive
P.O. Box 12233
Research Triangle Park, NC 27709

RE: Request for Additional Information on Toxicological Study Nominations to the National Toxicology Program (70 Federal Register 23877): Imidazolidinyl Urea

Dear Dr. Masten,

The Cosmetic, Toiletry, and Fragrance Association[1] (CTFA) appreciates the opportunity to provide additional information on the above referenced topic. Imidazolidinyl Urea is used as a preservative within the personal care products industry, and thus its nomination for study is of interest to CTFA members. When reading the NTP background document on Imidazolidinyl Urea, we noted that data on Diazolidinyl Urea are also included. The background document cites the Cosmetic Ingredient Review (CIR) as finding that Diazolidinyl Urea was not mutagenic in *S. typhimurium* and that it does not induce micronuclei, but that no technical details were available.

The three unpublished studies regarding genotoxicity of Diazolidinyl Urea cited in the CIR report are enclosed. These papers are listed as follows in the reference section of the CIR report.

33. Microbiological Associates. (July 29, 1983). Salmonella/mammalian-microsome mutagenicity assay. Submission of unpublished data by CTFA.

34. Microbiological Associates (September 15, 1983). Salmonella/mammalian-microsome mutagenicity assay. Submission of unpublished data by CTFA.

35. Pharmakon Research International, Inc. (December 12, 1986). Micronucleus test. Submission of unpublished data by CTFA.

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[1]Based in Washington, D.C., CTFA is the trade association representing the cosmetic, toiletry, and fragrance industry in the United States and globally. Founded in 1894, CTFA has a membership of nearly 600 companies including manufacturers, distributors, and suppliers for the vast majority of finished personal care products marketed in the United States.

Unpublished data cited in CIR reports are available from CIR upon request. In the future, if unpublished data cited in CIR reports are needed by the NTP, please contact Dr. Carol Eisenmann, at CTFA (eisenmannc@ctfa.org) and she will assist you in getting this information.

Sincerely,

Gerald N. McEwen, Jr., Ph.D., J.D.
Vice President - Science

Subject: Imidazolidinyl Urea
Date: Monday, June 6, 2005 10:16 AM
From: Carol Eisenmann <eisenmannc@ctfa.org>
To: <masten@niehs.nih.gov>

Dear Dr. Masten,

RE: Request for Additional Information on Toxicological Study Nominations to the National Toxicology Program (70 Federal Register 23877): Imidazolidinyl Urea

Attached, please find a submission letter and three unpublished studies on Diazolidinyl Urea mentioned in the Imidazolidinyl Urea background document.

This submission was also sent to you by FedEx on Friday June 3, 2005.

Sincerely,

Carol

Carol J. Eisenmann, Ph.D., D.A.B.T.
Toxicologist, Science Department
CTFA
1101 17th Street, N.W., Suite 300
Washington DC 20036-4702
Phone: 202 331-1770
Fax: 202 331-1969
eisenmannc@ctfa.org

GERMALL II

MOUSE MICRONUCLEUS TEST FOR CHROMOSOMAL ABERRATIONS

PH 309-SU-001-86

SUTTON LABORATORIES INC.
CHATHAM, N. J. 07928

PHARMAKON RESEARCH INTERNATIONAL, INC.
WAVERLY, PENNSYLVANIA 18471

PHONE
(717) 586-2411

Micronucleus Test (MNT) EPA

PH 309-SU-001-86

Germall II
Lot # GT-152

Submitted to

Sutton Laboratories, Inc.
Chatham, New Jersey

Ruth M. Sorg
Ruth M. Sorg, M.S. (Hyg.)
Study Director

December 10, 1986
Date

Robert W. Naismith
Test Facility Management

December 14, 1986
Date

TABLE OF CONTENTS

Micronucleus Test (MNT)

PH 309-SU-001-86
Germall II

SUMMARY

Doses for the Micronucleus Test on Germall II were selected from the previously reported results of an acute oral toxicity study in mice on Germall II done at Pharmakon Research International, Inc. In discussion with the sponsor, the doses selected were 1200, 2000 and 2800 mg/kg.

In the Micronucleus Test, nine groups of ten animals (5 males and 5 females/group) were given single doses of Germall II in 0.25% methylcellulose (0.25% MC) by oral gavage at 1200, 2000 or 2800 mg/kg and sacrificed at 30, 48 or 72 hours. Similar groups, administered the vehicle control, 0.25% MC were evaluated concurrently at each sacrifice interval. An additional group of ten animals (5 males and 5 females) was administered cyclophosphamide (CP) in 0.9% saline at a dose of 60 mg/kg and sacrificed at 30 hours, serving as the positive control. Slides were prepared from the bone marrow of the femora and stained. Coded slides were scored for the number of polychromatic erythrocytes (PCE) with micronuclei in 1000 PCE/animal. The ratio of polychromatic to normochromatic erythrocytes (NCE) per 1000 erythrocytes was determined for each animal.

The results for test article, Germall II, were negative in the Micronucleus Test at dose levels of 1200, 2000 and 2800 mg/kg at all of the time intervals evaluated. These findings are based upon the inability of the test article to produce a statistically significant increase in the number of micronuclei in 1000 polychromatic erythrocytes per animal in the treated groups versus the vehicle control groups.

STUDY DESCRIPTION

Sponsor: Sutton Laboratories
Chatham, New Jersey 07928

Study Number: PH 309-SU-001-86

TEST ARTICLE

The test article, Germall II, Lot # GT-152, was received by Pharmakon Research International, Inc. on July 24, 1986 in a plastic bag and the physical description of the test article upon receipt was a white powder. Normal precautions were used in handling the test article. All documentation supplied by the sponsor concerning stability and purity of the test article was the responsibility of the sponsor. Information as to the stability, purity, expiration date and other technical aspects of the test article was recorded in the sponsor's file. For the purposes of this study, the test article was stored at room temperature in the container received from the sponsor. All required dose levels were made with 0.25% methylcellulose on the day of administration. At the time of testing the test article was described as a white powder. There was no apparent change in the physical state of the test or control articles during the assay. Details of the test article preparation are contained in the Methods and Materials section of this report. Dosing solutions were used within two hours of preparation. Samples of the test article/vehicle solutions were sent to the sponsor for analysis, along with a sample of the vehicle control.

Date Micronucleus Test Initiated: October 21, 1986

Date Micronucleus Test Completed: November 21, 1986

Sponsor's Study Monitor: Grover Vernon Foster, Jr., Ph.D.

Pharmakon's Study Director: Ruth M. Sorg, M.S. (Hyg.), Pharmakon Research International, Inc.

Technical Performance: Nancy Gongliewski, Nira Madison, Susan M Lucenti, B.S., and Ruth M. Sorg, M.S. (Hyg.)

Notebook Reference: Notebook #1123, pages 88-131 (Study)

Good Laboratory Practices Statement: This study was conducted in compliance with the Good Laboratory Practice Regulations for non-clinical laboratory studies as developed by the U.S. Food and Drug Administration (Code of Federal

Regulations, Title 21, part 58 revised as of April 1, 1980), as well as the U.S. Environmental Protection Agency (EPA) as stated in the Federal Register, Vol. 48, No. 230, Tuesday, November 29, 1983 as well as the Organisation for Economic Co-operation and Development Guidelines for Testing Chemicals (OECD), ISBN 92-64-12221-4, adopted by the council at its 535th meeting on 12th May 1981. There were no significant deviations from the GLP Regulations which affected the quality or integrity of the study. Q.A.U. findings derived from the inspection(s) during the conduct of this study and from the audit of the final report are documented and have been provided to the study director and the test facility management.

Records Maintained: All correspondence pertinent to the study between the sponsor and Pharmakon Research International Inc., protocol, amendments to the protocol, raw data, test substance weight or volume, dispensation reports, quality assurance reports, the final report as well as microscope slides scored in the study are maintained in the Pharmakon Research International, Inc. Archives.

Test Facility Standard Operating Procedures: The methods utilized in this study are maintained in appropriate SOPs at Pharmakon Research International, Inc. and include SOPs PH-309, PH-007, PH-010, PH-020, PH-516, PH-545, PH-562 and PH-569.

PURPOSE

The purpose of this assay was to evaluate the potential of Germall II to induce micronuclei in mice pretreated by oral gavage with the test article. The Micronucleus Test detects in vivo damage to the chromosomes or mitotic apparatus by determining the presence of micronuclei in the polychromatic erythrocytes (PCE) in the bone marrow of mice. During development of an erythrocyte, the nucleus of the erythroblast is extruded and acentric fragments or lagging chromosomes may remain in the cells and become micronuclei. The cell then proceeds through a transient stage, the polychromatic erythrocyte which stains a bluish color as compared to the pink of the more mature normochromatic erythrocytes. Since micronuclei arise from chromosome fragments or chromosomes that are not incorporated into daughter nuclei at the time of cell division, the assay detects both clastogens and agents that affect the spindle apparatus(1).

Justification of Test System: Mice have historically been used in the Micronucleus Test and have been shown to exhibit micronuclei indicative of chromosome breakage(2) or lagging chromosomes. Oral administration was chosen as an acceptable alternative route to the IP route and more closely approximates the anticipated route of human exposure.

METHODS AND MATERIALS

Preparation of Control and Test Articles: Test article, Germall II, was weighed and diluted with 0.25% methylcellulose, Lot # 705259, [supplied by Fisher Scientific] (prepared in deionized water at Pharmakon Research International, Inc.). Good solutions were obtained and maintained at all levels evaluated utilizing a magnetic stir plate. The positive control, cyclophosphamide, was dissolved in 0.9% saline (Lot # 83-701-DM-01; Abbott Laboratories) and administered at 10 ml/kg at a dose of 60 mg/kg of body weight. Test article, Germall II, was administered at a dose volume of

10 ml/kg in the Micronucleus Test. The test article and positive control solutions were prepared fresh and dosed within two hours of preparation. There were no impurities expected to have been present in the vehicle control which would have affected the outcome of this assay.

Positive Control Article: Cyclophosphamide, Lot # 114F-0393
Source: Sigma Chemical Company

Dose Selection: Doses were selected for evaluation in the Micronucleus Test based on the previously reported results of an acute oral toxicity study[1] in mice on Germall II done at Pharmakon Research International, Inc. In discussion with the sponsor, doses selected for evaluation in the Micronucleus Test were 1200, 2000 and 2800 mg/kg.

MICRONUCLEUS TEST

Treatment Procedure(3,4): Seven week old CD-1 mice (male and female) were used in the study and supplied by Charles River Laboratories, Wilmington, Massachusetts. Animals were acclimated to laboratory conditions for five days prior to initiation of the assay. The albino mice were randomized by body weight and assigned to groups by use of a random number table and ear tagged. Initial body weights for the males ranged from 26-31 grams and females from 23-29 grams. Mice were housed five (5) per cage according to sex and dose group in stainless steel wire mesh cages in accordance with the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Resources, National Research Council. Waste material was removed three times per week. Throughout the study, animals were fed Wayne Rodent Blox and fresh tap water was also available ad libitum. Water is monitored for contaminants at periodic intervals and the results kept on file at Pharmakon Research International, Inc. No contaminants in the food and water were expected to have been present to interfere with the outcome of the study. The experimental design(5) for the Micronucleus Test was as follows:

Group	Dose	Animals per sacrifice time		
		30 hrs.	48 hrs.	72 hrs.
0.25% methylcellulose	10 ml/kg	10	10	10
Germall II	1200 mg/kg	10	10	10
Germall II	2000 mg/kg	10	10	10
Germall II	2800 mg/kg	10	10	10
Cyclophosphamide	60 mg/kg	10	-	-

The test article was administered in single oral doses to nine groups of ten animals (5 males and 5 females/group) at 1200, 2000 or 2800 mg/kg at 10 ml/kg which were sacrificed at 30, 48 and 72 hours. Concurrently cyclophosphamide in 0.9% saline, the positive control, was administered by oral gavage to mice (5 males and 5 females) at a dose of 60 mg/kg. Thirty hours after treatment, the positive control animals were sacrificed. Groups of ten animals (5 males and 5 females) were administered the vehicle control by oral gavage at

[1]PH 403-SU-001-86, October 14, 1986

10 ml/kg and sacrificed at 30, 48 and 72 hours. The time of sacrifice and cell harvest were determined from the time of treatment. All animals were sacrificed by cervical dislocation and their femora removed.

Pharmacotoxic Effects of Treatment: Animals were observed for mortality and pharmacotoxic signs immediately after dosing and at 4, 24, 48 and 72 hours after dose administration when applicable.

Animals administered Germall II at 1200 mg/kg exhibited no signs immediately after dosing. At 4, 24, 48 and 72 hours one or more animals exhibited decreased body tone. Abnormal gait was also observed occasionally at 4 and 24 hours.

At 2000 mg/kg decreased body tone was observed in several animals at all observation times. Abnormal gait was also observed in six males and three females at 24 hours.

At 2800 mg/kg decreased body tone was observed in several animals at all observation times. Approximately two-thirds of the animals at this level also exhibited abnormal gait at 4 and 24 hours. Additional signs observed at this level included vocalization to the touch in two females and one male at 4 hours, and decreased activity in two males at 4 hours.

At 2800 mg/kg, a total of three animals (all males) died during the in vivo phase of the study. One male (# 4222 in the 72 hour sacrifice group) died by 7 hours after dose administration. Gross necropsy findings for this animal indicated fluid filled, distended intestines which were dark red in color. The glandular section of the stomach contained hemorrhogic areas and was distended and the stomach was filled with clear fluid. By 24 hours two additional males were dead (# 4184 in the 48 hour sacrifice group and # 4225 in the 72 hour sacrifice group). Gross necropsy finding for # 4184 revealed distended intestines filled with red fluid and a distended stomach filled with clear fluid. The glandular section of the stomach was red in color and contained hemorrhogic areas. Gross necropsy of male # 4225 revealed a distended stomach filled with a clear fluid, and the glandular portion of the stomach was red in color. The lungs were also red in color.

No signs were observed in animals administered the positive or vehicle controls.

Slide Preparation: Both femora of each individual animal were opened carefully at the proximal end with a scissors until a small opening to the marrow canal became visible. A 1 ml tuberculin syringe filled with approximately 0.2 ml fetal bovine serum was inserted into the bone and the bone marrow was gently flushed (to assure maximum dispersion) into 1.0 ml of fetal bovine serum in a 3 ml conical centrifuge tube. The femora were flushed with fetal bovine serum until all the marrow was out and the bone appeared almost transparent. If necessary, the distal ends were opened and flushed. Bone marrow suspensions from both femora of each individual animal were pooled and treated as a single sample for slide preparation. The suspension was

centrifuged at 1000 rpm in a Sorvall RC-5 centrifuge with an HS-4 head for five minutes. The supernatant was removed leaving a small amount of fetal bovine serum with the remaining cell button. The button was mixed with a pasteur pipette to assure a homogenous mixture. A small drop of the mixture was immediately placed near the frosted end of a glass microscope slide previously cleaned in absolute ethanol and pulled behind a clean slide at a 45° angle. The slides were quick dried on a slide warmer set at approximately 56°C. Following preparation of the smears, they were dipped in absolute methanol and allowed to air dry.

Staining: The slides were stained according to the following procedure:

a) Fix in absolute methanol - 5 minutes and air dry.
b) Remove metallic film from surface of Giemsa working solution (5% Giemsa in pH 6.8 phosphate buffer solution) using a paper towel.
c) Stain 20 minutes in Giemsa working solution.
d) Rinse twice. The first rinse is in deionized water adjusted to pH 4.0 to 4.5 and the second rinse in deionized water adjusted to pH 7.0.
e) Clean back of slide with absolute methanol.
f) Dry on 56°C slide warmer.
g) Clear in xylene.
h) Mount in Permount with cover glass.

Coding of Slides: Slides were coded randomly by study number and number designation. The code was kept on a separate sheet in the sponsor's file until the slides were evaluated. Following evaluation, the slides were decoded and the code sheet was placed in the notebook. The coding of the slides was carried out by an individual not involved in the actual scoring of the study.

Criteria for Scoring Micronuclei: Micronuclei are uniform, darkly stained, typically round bodies in the cytoplasm of PCE. Occasionally, micronuclei appear almond or tear drop shaped. Inclusions in PCEs which were reflective, improperly shaped or stained, or which were not in the focal plane of the cell were judged to be artifacts and were not scored as micronuclei. Cells containing more than one micronucleus were only scored as one micronucleated PCE.

Slide Analysis: The slides were screened for good preparation, i.e. well spread, undamaged, perfectly stained. One thousand (1000) PCE per animal were counted for the presence of micronuclei. The data were expressed as the number of micronucleated PCE versus total normal PCE in 1000 total PCE per animal (Tables 1, 2 and 3).

A total of 1000 polychromatic and normochromatic erythrocytes (NCE) was also counted per animal. These data were expressed as the ratio of polychromatic erythrocytes to normochromatic erythrocytes (Tables 4, 5 and 6). For each group of ten animals designated in each dose group (Tables 1-6), male numbers 1-5 represent the first to the fifth male. Female numbers 6-10 represent the first to the fifth female in each respective group.

Statistical Evaluation: Assessment of a test article as positive is based upon its ability to produce a statistically significant increase in the number of micronucleated polychromatic erythrocytes as compared to the vehicle control. One-tailed t tests were used to make pairwise comparisons between each treatment group and its concurrent vehicle control for statistically significant increases in the number of micronucleated PCE. The ratio of polychromatic to normochromatic erythrocytes was also calculated based on 1000 erythrocytes for each animal. The proportion of polychromatic erythrocytes per 1000 erythrocytes per animal was evaluated by pairwise t tests after an arcsin transformation was performed. Statistical significance was judged at $p \leq 0.05$ and $p \leq 0.01$ levels. All comparisons were made for each sacrifice time separately comparing treated groups versus the vehicle control group.

Results: No statistically significant increases in the incidence of micronucleated PCE were detected in animals treated with Germall II at any of the sacrifice times evaluated.

A statistically significant elevation in the PCE/NCE ratio was detected in animals administered Germall II at 2800 mg/kg and sacrificed at 72 hours ($p \leq 0.05$). The significance of elevations in this ratio is unclear.

Animals treated with the positive control gave a statistically significant increase in the incidence of micronucleated PCE ($p \leq 0.01$) and a statistically significant ($p \leq 0.05$) depression of the PCE/NCE ratio.

Criteria for a Valid Test: If the spontaneous rate of micronuclei in the polychromatic erythrocytes is less than 0.5% and the positive control is statistically greater ($p \leq 0.05$) than the spontaneous and at least seven animals per group survived the treatment, the results will be deemed acceptable. This study fulfilled the criteria of a valid test.

CONCLUSION

The results for test article, Germall II, were negative in the Micronucleus Test at dose levels of 1200, 2000 and 2800 mg/kg of body weight administered in single oral doses with sacrifice times of 30, 48 and 72 hours. These findings are based upon the inability of the test article to produce a significant increase in the incidence of micronuclei per 1000 polychromatic erythrocytes per animal in the treated groups versus the vehicle control groups under the conditions of this assay.

REFERENCE:

1. Heddle, J.A., M. Hite, B. Kirkhart, K. Mavournin, J. T. MacGregor, G.W. Newell and M.F. Salamone. Induction of Micronuclei as a Measure of Genotoxicity. A Report of the U.S. Environmental Protection Agency Gene-Tox Program, 1983, Volume 123, pages 61-118.

2. Schmid, W., The Micronucleus Test, Mutation Research, 31 (1975) 9-15.

3. EPA New and Revised Health Effects Test Guidelines, Federal Register Vol. 50, No. 188, Friday, September 27, 1985.

4. Organisation for Economic Co-operation and Development Guidelines for Testing Chemicals (OECD), ISBN 92-64-12221-4, adopted by the council at its 535th meeting on 12th May, 1981.

5. Salamone, M., J. Heddle, E. Stuart and M. Katz. Towards An Improved Micronucleus Test, Mutation Research 74 (1980) 347-356.

Table 1

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Micronucleated PCE/1000 Polychromatic Erythrocytes/Animal

Animal Number	Controls		30 hour sacrifice		
	0.25% MC	CP (30 hr.)	Germall II		
Male	10 ml/kg	0.5 mg/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	3	44	1	2	1
2	0	24	0	0	0
3	2	93	1	0	2
4	2	27	1	0	2
5	0	17	0	1	1
Female					
6	1	19	5	4	0
7	1	26	3	2	3
8	1	24	1	1	1
9	3	30	2	1	1
10	1	50	0	0	0
Mean ±S.D.	1.40±1.07	35.40±22.76	1.40±1.58	1.10±1.29	1.10±0.99
t value	-	4.719**	0	0.565	0.647

** Denotes statistical significance at $p \leq 0.01$.

Table 2

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Micronucleated PCE/1000 Polychromatic Erythrocytes/Animal

Animal Number	Controls	48 hour sacrifice		
	0.25% MC	Germall II		
Male	10 ml/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	3	1	0	0
2	2	2	3	0
3	0	3	1	2
4	1	3	0	Dead
5	2	1	3	3
Female				
6	1	1	1	2
7	1	1	3	1
8	1	0	0	5
9	0	0	1	1
10	0	0	1	0
Mean ±S.D.	1.10 ± 0.99	1.20 ± 1.14	1.30 ± 1.25	1.56 ± 1.67
t value	-	0.209	0.395	0.732

Table 3

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Micronucleated PCE/1000 Polychromatic Erythrocytes/Animal

Animal Number	Controls	72 hour sacrifice		
	0.25% MC	Germall II		
Male	10 ml/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	0	4	1	1
2	1	0	2	Dead
3	2	0	0	0
4	4	1	1	0
5	0	0	0	Dead
Female				
6	1	0	1	0
7	0	1	2	1
8	0	2	1	1
9	1	0	1	0
10	1	0	1	0
Mean ±S.D.	1.00 ± 1.25	0.80 ± 1.32	1.00 ± 0.67	0.38 ± 0.52
t value	-	0.348	0	1.322

Table 4

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Ratio of Polychromatic Erythrocytes to Normochromatic Erythrocytes in 1000 Erythrocytes/Animal

	Controls		30 hour sacrifice		
Animal Number	0.25% MC	CP(30 hr.)	Germall II		
Male	10 ml/kg	60 mg/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	0.429	0.984	1.057	0.828	0.919
2	1.469	0.869	1.257	0.984	1.008
3	0.639	0.634	1.283	1.681	1.222
4	0.957	0.672	1.008	1.551	1.375
5	1.825	0.603	0.541	1.611	1.404
Female					
6	1.353	1.183	2.597	2.145	2.436
7	1.882	1.137	2.289	1.294	1.273
8	1.174	0.876	2.831	1.857	2.030
9	1.481	1.123	1.169	1.415	1.625
10	1.710	1.165	1.809	2.401	1.336
Mean ±S.D.	1.29±0.49	0.92±0.23	1.58±0.76	1.58±0.48	1.46±0.46
t value[a]	-	2.243*	0.451	0.918	0.536

* Denotes statistically significant depression at $p \leq 0.05$.

[a] t values are derived from comparisons between groups using the arcsin transformed value for the proportion of PCE for each animal and not from the tabulated valued for ratios given in this table.

Table 5

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Ratio of Polychromatic Erythrocytes to Normochromatic Erythrocytes in 1000 Erythrocytes/Animal

Animal Number	Controls	48 hour sacrifice		
	0.25% MC	Germall II		
Male	10 ml/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	1.232	1.262	0.908	1.571
2	1.433	1.874	1.128	1.146
3	1.639	1.500	1.415	1.907
4	1.778	2.096	1.410	Dead
5	2.106	2.145	1.174	1.506
Female				
6	1.273	1.358	1.551	1.632
7	1.193	1.793	2.086	1.747
8	1.558	1.653	1.445	1.392
9	2.247	1.985	1.364	2.096
10	1.564	1.959	1.488	1.564
Mean ±S.D.	1.60 ± 0.36	1.76 ± 0.31	1.40 ± 0.31	1.62 ± 0.28
t value[a]	-	1.144	1.413	0.204

[a] t values are derived from comparisons between groups using the arcsin transformed value for the proportion of PCE for each animal and not from the tabulated values for ratios given in this table.

Table 6

Micronucleus Test
SUMMARY DATA

PH 309-SU-001-86

Ratio of Polychromatic Erythrocytes to Normochromatic Erythrocytes in 1000 Erythrocytes/Animal

Animal Number	Controls	72 hour sacrifice		
	0.25% MC	Germall II		
Male	10 ml/kg	1200 mg/kg	2000 mg/kg	2800 mg/kg
1	1.294	1.398	1.770	1.237
2	0.912	1.242	1.353	Dead
3	1.959	1.294	0.838	0.912
4	1.070	1.053	1.165	1.232
5	0.949	1.262	1.506	Dead
Female				
6	1.732	1.463	0.988	2.344
7	1.740	1.415	1.571	2.759
8	0.695	1.045	1.469	2.322
9	1.179	1.252	1.899	2.300
10	1.427	1.882	1.475	2.344
Mean ±S.D.	1.30 ± 0.41	1.33 ± 0.24	1.40 ± 0.33	1.93 ± 0.69
t value	-	0.534	0.736	2.137*

* Denotes statistically significant elevation in PCE/NCE ($p \leq 0.05$).

[a] t values are derived from comparisons between groups using the arcsin transformed value for the proportion of PCE for each animal and not from the tabulated values for ratios given in this table.

PHARMAKON RESEARCH INTERNATIONAL, INC.
WAVERLY, PENNSYLVANIA 18471
PHONE
(717) 586-2411

QUALITY ASSURANCE UNIT STATEMENT

Study No.: PH 309-SU-001-86

Study Director: Ruth M. Sorg

This study was conducted in compliance with the Good Laboratory Practice Regulations. The Quality Assurance Unit conducted the inspections listed below and reported the results to the study director and to management on the dates indicated.

The following inspections were performed:

Interval	Date
In Vivo Preliminary Phase	10/21/86
Cell and Slide Preparation Phase	10/22/86
Reporting Phase	11/26/86

Date QAU Report Issued

To Study Director	To Management
11/26/86	11/26/86

11/26/86
Date

Quality Assurance

PHARMAKON RESEARCH INTERNATIONAL, INC.
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(717) 586-2411

Compliance Statement

This study was conducted in compliance with the Principles of Good Laboratory Practice (GLP) as promulgated by the following regulatory agencies:

U.S. Food and Drug Administration, as stated in the Code of Federal Regulations, Title 21, Part 58, revised as of April 1, 1980.

U.S. Environmental Protection Agency as stated in the Federal Register, Vol. 48, No. 230, Tuesday, November 29, 1983.

Organization for Economic Co-operation and Development Guidelines for Testing Chemicals (OECD), ISBN 92-64-12221-4, adopted by the council at its 535th meeting on 12th May, 1981.

Study No.: PH 309-SU-001-86

"To the best of my knowledge, the study was conducted in accordance with applicable Good Laboratory Practice regulations; there were no deviations from these regulations that impacted on study conclusions."

Ruth M. Sorg
Study Director

December 10, 1986
Date

Notebook # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

Genetic Toxicology
Study Assignment

Study Number: PH309-SU-001-86 Protocol Available (yes) no (circle)

Study Description: Cytogenetic Assay - Micronucleus Test

Study Director: Ruth M. Sorg M.S.(Hyg) PRI

Technical Performance: Nancy Gonglieuski, Nira Madison, Susan M. Lucente B.S. and Ruth M. Sorg M.S.(Hyg.)

Standard Operating Procedure: PH309

Sponsor: Sutton Laboratories, Inc
Chatham, New Jersey 07928

Monitor: Grover V. Foster, Jr., Ph.D.

Test Article: Germall II Lot No. GT-152

Physical Description: White Powder

Date of Study Assignment from Quality Assurance: 10-9-86

Study Initiation: 10-21-86

Study Completion: 11-21-86

Solubility: Soluble in 0.25% methylcellulose

Sponsor's Suggestion? (yes) no (circle)

Solubility Assay Conducted: yes (no) (circle)

Vehicle (Solvent): 0.25% methylcellulose

Preliminary Toxicity Form #: Not applicable

Comments: Methylcellulose Lot #705259 - Compound prep 0.25% MC
1.25gm methylcellulose + / RM 10/17/86
500 ml DH2O / Expiration date 10/31/86

Study Director: Ruth M. Sorg Date: 11-21-86

CHTRMS(6)

Notebook # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc Date Initiated: 10-21-86
Test Article: Grenade II Date Terminated: 11-21-86
Description: White Powder

Purpose: To evaluate the potential of the test article to induce micronuclei when administered to mice.

Dose Levels Evaluated: Based on the acute oral toxicity study in mice done at PRI, and in discussion with the sponsor, doses selected for evaluation were 1200, 2000 and 2800 mg/kg.

Sacrifice Times: 30, 48 and 72 hours

Vehicle: 0.25% methylcellulose Lot # 705259 Source: Fisher Scientific
Volume Administered: 10 ml/kg
Route of Administration: Oral
Fasting: Approximately 4 hours before dosing. Food returned immediately after dosing. ——→ 5AM - CP 41012-41109
5:30AM MC 30, 48, 72hr
6 AM 1200 mg/kg 30, 48, 72hr.
6:30AM 2000 mg/kg 30, 48, 72hr
7 AM 2800 mg/kg 30, 48, 72 hr.

Species: Mouse Strain: CD-1 16 20 RMS 11-25-86
Animal P.O.# 041585 F TOX Date Rec'd: 10/16/86
Age at initiation: 7 weeks Birth Date: 8/31/86
No. of Animals per group: 10 Males 5 Females 5
Randomization: By weight and random number Table.
Animal Identification: Ear tagged and steel cages marked.

Food Lot # PO 9166 Date Rec'd: 10-7-86 Type: Wayne Rodent Chow
Animal Scale #: Sartorius 8 Calibrated: daily
Study Room # Tox III
Light Cycle Checked: 10/21/86 RMS 12 hrs. light, 12 hrs. dark
10/22/86 RMS
10/23/86 RMS
10/24/86 RMS
Temperature and humidity: Constantly monitored and recorded with a Honeywell Relative Humidity and Temperature Recorder Model 61, week of 10/16/86 - 10/22/86 and 10/23/86 - 10/29/86

Tracey Gongleweski 11/21/86
Investigator Date

Fetal Bovine Serum:
30 hr groups: Lot# KC 300449 10-22-86 RMS
48hr " "
72hr " " Lot KC 300504

Mark M. Sorg 11-21-86
Study Director Date

CHTRMS(5)

Notebook # 1123

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. SH309-SU-001-8

Sponsor: Sutton Laboratories, Inc. Date Initiated: 10-21-86

Test Article: Germall II Date Terminated: 11-21-86

Description: White Powder

TEST ARTICLE PREPARATION Germall II Lot #GT-152

PLS 10-21-86 1200 mg/kg @ 10 ml/kg → 120 mg/ml
2400 mg Germall II qs to 20 ml with 0.25% methylcellulose

PLS 10-21-86 2000 mg/kg @ 10 ml/kg → 200 mg/ml
4000 mg Germall II qs to 20 ml with 0.25% methylcellulose

PLS 10-21-86 2800 mg/kg @ 10 ml/kg → 280 mg/ml
5600 mg Germall II qs to 20 ml with 0.25% methylcellulose

Compound Preparation Scale # Sartorius II 1602 mp Calibrated: 10-21-86

Comments: Test article weighed in mortar (tared to 0.000 gm) and a small amount of 0.25% mc was added to suspend the compound. Suspension transferred to pharmaceutical graduated cylinder and qs to the appropriate volume with 0.25% mc. Suspension/solution vortex mixed and immediately transferred to original mortar for dosing. Mixture continuously stirred on magnetic stir plate during dosing phase PLS 10-21-86. An aliquot sample of each dose was taken immediately after preparation - PLS 10-21-86

Samples sent: (yes) no

Date sent: 10/21/86

Dose preparation formed: A solution at all levels evaluated

Total Germall II used: 12.0 gms 10-21-86 PLS

Nancy Dongkowski 11/21/86
Investigator Date

Ruth M. Sarg 11-21-86
Study Director Date

Page 90

CHTRMS(1)

Notebook # 1123

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc. Date Initiated: 10-21-86

Test Article: Germall II Date Terminated: 11-21-86

Description: White powder

TEST ARTICLE PREPARATION - Positive Control

Cyclophosphamide (CP) @ 60mg/kg @ 10ml/kg → 6 mg/ml.

6.7338 gm vial + CP
plus
10-21-86 6.6430 gm vial
0.0908 gm CP = 90.8 mg CP

$\frac{90.8 \text{ mg CP}}{6 \text{ mg/ml}}$ = 15.1 ml 0.9% Saline.

CP Lot # 114F-0393 Sigma Chemical Co.
0.9% Saline Lot # 83-701-DM-01 Abbott Laboratories, North Chicago Illinois

Compound Preparation Scale # Sartorius V Calibrated: 10/21/86
16.02 mg

Comments: CP weighed in tared glass vial and diluted to appropriate volume with 0.9% Saline in 50 ml tube.

Samples sent: yes (no)
Date sent: N/A
Dose preparation formed: Solution

Nancy Jaroszewski 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test | STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories | Date Initiated: 10/21/86

Test Article: Gumsol II | Date Terminated: 11-21-86

Description: White Powder | Dose Level: 60mg/kg @10ml/kg → 6 mg/ml

Observations: Immediate:

10/10 animals no signs SHW 10-21-86
4hrs pd: 10/10 no signs NB 10/21/86
24hrs pd: 10/10 no signs NB 10/22/86

DOSE: Cyclophosphamide 60mg/kg SAMPLE TIME: 30 Hrs

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4101	♂	29	.29	9[10] AM	32	10/22/86	956	44
4102	♂	30	.30	↓	32		976	24
4103	♂	27	.27		27		907	93
4104	♂	29	.29		30		973	27
4105	♂	28	.28		30		983	17
4106	♀	24	.24		25		981	19
4107	♀	24	.24		25		974	26
4108	♀	24	.26		26		976	24
4109	♀	28	.28		29		970	30
4110	♀	24	.26	9[15] AM	26		950	50
		NM 10/21/86	NM 10/21/86	SHW 10-21-86	RMS 10-22-86	RMS 10-22-86	NM 11/21/86	NM 11/21/86

Dosed by: Nira Madison 10/21/86

Mean: 35.400 ± 22.755

t Values: 4.719** p 10E NM 11/21/86

** Denotes statistical significance at p

Nancy Ekrzyiewski 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

Page

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH 309-SU-001-86

Sponsor: Sutton Laboratories Date Initiated: 10/21/86

Test Article: Seimelg II Date Terminated: 11-21-86

Description: White Powder Dose Level: @ 10 ml/kg

DOSE: MC SAMPLE TIME: 30 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4111	♂	30	.30	9 30	32	10	997	3
4112	♂	29	.29		31		1000	0
4113	♂	29	.29		31		998	2
4114	♂	29	.29		31		998	2
4115	♂	31	.31		32	22	1000	0
4116	♀	27	.27		28		999	1
4117	♀	25	.25		26		999	1
4118	♀	26	.26		28	86	999	1
4119	♀	24	.24	↓	25		997	3
4120	♀	26	.26	9 33	27		999	1
		JM 10/21/86	JM 10/21/86	LHL 10-21-86	RMS 10-22-86	RMS 10-22-86	JM 11/21/86	RMS 11-21-86

Dosed by: Mira Madison 10/21/86

Observations: Immediate:

10/10 animals - no signs 10-21-86

4 hrs pd: 10/10 animals no signs 10/21/86 NB

24 hrs pd: 10/10 no signs NB 10/22/86

Mean: 1.400 ± 1.074

t Values:

Nancy Houghton-Allen 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories

Test Article: Germall II

Description: White Powder

Date Initiated: 10-21-86

Date Terminated: 11-21-86

Dose Level: 1200 mg/kg @ 10 ml/kg → 120 mg/ml

DOSE: 1200 mg/kg SAMPLE TIME: 30 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4121	♂	31	.31	10 AM	32		999	1
4122	♂	30	.30		31	10/22/86	1000	0
4123	♂	28	.28		28		999	1
4124	♂	31	.31		32		999	1
4125	♂	29	.29		31		1000	0
4126	♀	25	.25		25		995	5
4127	♀	25	.25		25		997	3
4128	♀	26	.26		26		999	1
4129	♀	25	.25		26		998	2
4130	♀	27	.27	10 AM	27		1000	0
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	RHS 10-22-86	RHS 10-22-86	nm 11-21-86	nm 11-21-86

Dosed by: Ala Madison 10/21/86

Observations: Immediate:

10/10 animals - no signs AHL 10-21-86

4 hrs pd: 1/5♂ decreased body tone 4/5♂ 5/5♀ no signs 10/21/86 NS

24 hrs pd: 1/5♂ 1/5♀ decreased body tone 4/5♂ ~~2/5~~ 4/5♀ no signs R, DENG 10/22/86

Mean: 1.400 ± 1.577

t Values: 0

Nancy Gorzlewick 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories Date Initiated: 10-21-86

Test Article: Gemnall III Date Terminated: 11-21-86

Description: White Powder Dose Level: 2000 mg/kg @ 10 ml/kg → 200 mg/ml

DOSE: 2000 mg/kg SAMPLE TIME: 30 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4131	♂	27	.27	10[32] AM	29	10/22/86	998	2
4132	♂	29	.28		28		1000	0
4133	♂	29	.29		30		1000	0
4134	♂	30	.30		31		1000	0
4135	♂	30	.30		30		999	1
4136	♀	23	.23		24		996	4
4137	♀	27	.27		27		998	2
4138	♀	27	.27		27		999	1
4139	♀	26	.26	↓	26		999	1
4140	♀	28	.28	10[37] AM	28		1000	0
		DHL 10-21-86	DHL 10-21-86	DHL 10-21-86	RHS 10/22/86	RHS 10/22/86	11/21/86	11/21/86

Dosed by: Nita Madison 10/21/86

Observations: Immediate:

1/5 ♂ decreased body tone
4/5 ♂ no signs NB 10/22/86
1/5 ♀ decreased body tone
4/5 ♀ no signs DHL 10-21-86
4 hrs pd: 1/5 ♂ 1/5 ♀ decreased body tone 4/5 ♂ 4/5 ♀ no signs NB 10/22/86
24 hrs pd: 1/5 ♂ decreased body tone, abnormal gait 4/5 ♂ no signs
1/5 ♀ decreased body tone 4/5 ♀ no signs
NB 10/22/86

Mean: 1.100 ± 1.286

t Values: 0.565

Nancy Gezgiewski 11/21/86
Investigator Date

Kurt M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories

Test Article: Germall II

Description: White Powder

Date Initiated: 11-21-86

Date Terminated: 11-21-86

Dose Level: 2800 mg/kg @ 10 ml/kg → 280 mg/ml

DOSE: 2800 mg/kg SAMPLE TIME: 30 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4141	♂	27	.27	10 11 AM	28		999	1
4142	♂	30	.30		31	10/	1000	0
4143	♂	29	.29		30	22/	998	2
4144	♂	28	.28		28		998	2
4145	♂	31	.31		32	86	999	1
4146	♀	27	.27		28		1000	0
4147	♀	25	.25		24		997	3
4148	♀	24	.24		25		999	1
4149	♀	25	.25		27		999	1
4150	♀	28	.28	12 11 AM	28		1000	0
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	RHS 10-22-86	AHS 10-22-86	NM 11-21-86	NM 11-21-86

Dosed by: Nita Madison 10/21/86

Observations: Immediate:

4/5 ♂ 4/5 ♀ no signs ; 1/5 ♂ 1/5 ♀ decreased body tone AHL 10-21-86

4hr p.d.: 4/5 ♂ decreased body tone + abnormal gait 2/5 ♂ abnormal gait 2/5 ♂ no signs 4/5 ♀ decreased body tone abnormal gait 1/5 ♀ abnormal gait 1/5 ♀ vocalization on touch 2/5 ♀ no signs ND 10/21/86

24hr p.d.: 3/5 ♂ decreased body tone abnormal gait 1/5 ♂ abnormal gait 1/5 ♂ decreased body tone 1/5 ♀ decreased body tone abnormal gait 1/5 ♀ decreased body tone 1/5 ♀ abnormal gait 2/5 ♀ no signs ND 10/22/86

Mean: 1.100 ± 0.994

t Values: 0.647

Nancy Gorzlewski 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test — STUDY NUMBER: PH 309-SU-001-86

Sponsor: Sutton Laboratories — Date Initiated: 10/21/86

Test Article: Germall II — Date Terminated: 11-21-86

Description: White Powder — Dose Level: @ 10 ml/kg

DOSE: MC — SAMPLE TIME: 48 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4151	♂	31	.31	9 34 AM	34		997	3
4152	♂	28	.28		31	10/	998	2
4153	♂	30	.30		34		1000	0
4154	♂	30	.30		33	23/	999	1
4155	♂	30	.30		32		998	2
4156	♀	25	.25		27		999	1
4157	♀	26	.26		28	86	999	1
4158	♀	26	.26		29		999	1
4159	♀	28	.28		31		1000	0
4160	♀	27	.27	9 37 AM	29		1000	0
		NM 10/21/86	NM 10/24/86	AHL 10-21-86	NB 10/23/86	NG 10/23/86	NM 11/21/86	NM 11/21/86

Dosed by: Nia Madison 10/21/86

Observations: Immediate:

10/10 animals - no signs AHL 10-21-86
4 hrs pd: 10/10 no signs NB 10/21/86
24 hrs pd: 10/10 no signs NB 10/22/86
48 hrs pd: 10/10 no signs NB 10/23/86

Mean: 1.100 ± 0.994 (0.99 changed to 0.994, initialed NM, 11-21-86)

t Values:

Nancy Gongliewski 11/21/86
Investigator — Date

Ruth M. Sorg 11-21-86
Study Director — Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories
Test Article: Germall II
Description: White Powder

Date Initiated: 10-21-86
Date Terminated: 11-21-86
Dose Level: 1200 mg/kg @ 10 ml/kg → 120 mg/ml

DOSE: 1200 mg/kg SAMPLE TIME: 48 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4161	♂	26	.26	10[12] AM	29	|	999	1
4162	♂	31	.31		32	10/	998	2
4163	♂	29	.29		33		997	3
4164	♂	29	.29		33	23/	997	3
4165	♂	29	.29		31		999	1
4166	♀	26	.26		28		999	1
4167	♀	27	.27		28	86	999	1
4168	♀	26	.26		28		1000	0
4169	♀	27	.27	↓	27	|	1000	0
4170	♀	25	.25	10[16] AM	26		1000	0
		RHL 10-21-86	RHL 10-21-86	RHL 10-21-86	NB 10/23/86	NB 10/23/86	RM 11-21-86	RM 11-21-86

Dosed by: Nere Madison 10/21/86

Observations: **Immediate:**

10/10 animals - no signs RHL 10-21-86

4 hrs pd: 1/5♂ 4/5 (RHL 10/21/86) 1/5 abnormal gait 1/5♀ decreased body tone 3/5♀ no signs 1/5♂ decreased body tone 4/5♂ no signs NB 10/21/86

24 hrs pd: 2/5♂ decreased body tone abnormal gait 3/5♂ no signs 1/5 females decreased body tone abnormal gait 4/5♀ no signs NB 10/22/86

48 hrs pd: 5/5♂ 5/5♀ no signs NB 10/23/86

Mean: 1.200 ± 1.135

t Values: 0.209

Nancy Georgiesusko 11/21/86
Investigator /Date

Ruth M. Sorg 11-21-86
Study Director Date

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NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories

Test Article: Germall II

Description: White Powder

Date Initiated: 10-21-86

Date Terminated: 11-21-86

Dose Level: 2000 mg/kg @ 10 ml/kg → 200 mg/kg

DOSE: 2000 mg/kg SAMPLE TIME: 48 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4171	♂	29	.29	10:38 AM	31	10/23/86	1000	0
4172	♂	31	.31		33		997	3
4173	♂	29	.29		31		999	1
4174	♂	28	.28		32		1000	0
4175	♂	30	.30		33		997	3
4176	♀	25	.25		26		999	1
4177	♀	25	.25		28		997	3
4178	♀	~~26~~ 26 (ICE AHL 10-21-86)	.26		28		1000	0
4179	♀	25	.25		28		999	1
4180	♀	26	.26	10:42 AM	28		999	1
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	NG 10/23/86	NG 10/23/86	NM 11-21-86	NM 11-21-86

Dosed by: Nea Madison 10/21/86

Observations: Immediate:

1/5♂ 1/5♀ decreased body tone
4/5♂ 4/5♀ no signs AHL 10-21-86

4 hrs pd: 1/5♂ 1/5♀ decreased body tone
4/5♂ 4/5♀ no signs NG 10/21/86

24 hrs pd: 5/5♂ abnormal gait 1/5♂ decreased body tone 1/5♀ abnormal gait 2/5♀ abnormal gait decreased body tone 2/5♀ no signs NG 10/22/86

48 hrs pd: 3/5♂ 1/5♀ abn decreased body tone 2/5♂ 4/5♀ no signs NG 10/23/86 (1DE NG 10/23/86; 1DE NG 10/21)

Mean: 1.300 ± 1.251

t Values: 0.395

Nancy Gorglewski 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories Date Initiated: 10-21-86

Test Article: Gumsell II Date Terminated: 11-21-86

Description: White Powder Dose Level: 2800 mg/kg @ 10 ml/kg → 280 mg/ml

DOSE: 2800 mg/kg SAMPLE TIME: 48 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4181	♂	30	.30	11[15] AM	32	10/	1000	0
4182	♂	27	.27		29	23/	1000	0
4183	♂	29	.29		31	86	998	2
4184	♂	29	.29		28 Died	10/22/86	—	—
4185	♂	29	.29		31		997	3
4186	♀	26	.26		28	10/	998	2
4187	♀	25	.25		25	23/	999	1
4188	♀	28	.28		27	/	995	5
4189	♀	25	.25	↓	27	86	999	1
4190	♀	25	.25	11[17] AM	24	1	1000	0
		AHL 10-21-86	AHL 10-21-86	AHL 10 21-11	NG 11/23/86	NG 10/23/86	NG 11-21-86	NG 11-21-86

Observations: Immediate:

2/5♂ 3/5♀ decreased body tone no signs in remaining ♂♀ animals AHL 10-21-86

4hrspd: 1/5♂ abnormal gait, decreased body tone 1/5♂ abnormal gait, vocalization on touch 3/5♂ abnormal gait 5/5♀ abnormal gait 1/5♀ vocalization on touch, 1/5♀ decreased body tone NG 10/21/86

24 Hrs 10/22/86 4184♂ Necropsy Stomach distended/fluid filled (clear) Glandular section red in color hemorrhagic areas. Intestines distended fluid filled (red) NG

24hrspd 4/4♂ abnormal gait 1/5♀ decreased body tone 4/5♀ abnormal gait 1/5♀ decreased body tone 1/5♀ no signs NG 10/22/86

48hrspd: 3/4♂ 2/5♀ decreased body tone 1/4♂ 3/5♀ no signs NG 10/23/86

Mean: 1.555 ± 1.66

t Values: 0.732

Dosed by: Nina Madison 10/21/86

Nancy Gonglewski 11/21/86
Investigator Date

Kurt M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH.309-30-001-86

Sponsor: Sutton Laboratories Date Initiated: 10/21/86

Test Article: Germal II Date Terminated: 11-21-86

Description: White Powder Dose Level: @ 10 ml/kg

DOSE: MC SAMPLE TIME: 72 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4191	♂	29	.29	9[33] AM	33		1000	0
4192	♂	28	.28		30		999	1
4193	♂	29	.29		32	10/	998	2
4194	♂	30	.30		33	/24/	996	4
4195	♂	30	.30		34	/86	1000	0
4196	♀	25	.25		28		999	1
4197	♀	24	.24		26		1000	0
4198	♀	25	.25		23		1000	0
4199	♀	26	.26	↓	28		999	1
4200	♀	26	.26	9[42] AM DAL 10-21-86	27		999	1
		NM 10/21/86	NM 10/21/86		NM 10/24/86	NM 10/24/86	NM 11-21-86	NM 11-21-86

Observations: Immediate:

10/10 animals - no signs DAL 10-21-86

4 hrs pd: 10/10 no signs NS 10/21/86

24 hrs pd: 10/10 no signs NS 10/22/86

48 hrs pd: 10/10 no signs NS 10/23/86

72 hrs pd 10/10 no signs. NM 10/24/86

Mean: 1.000 ± 1.247

t Values:

Dosed by: Nina Madison 10/21/86

Nancy Gonglewski 11/21/86
Investigator Date

Dust M. Sorg 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH 309-SU-001-86

Sponsor: Sutton Laboratories

Test Article: Germall II

Description: White Powder

Date Initiated: 10-21-86

Date Terminated: 11-21-86

Dose Level: 1200 mg/kg @ 10 ml/kg → 120 ml/kg

DOSE: 1200 mg/kg SAMPLE TIME: 72 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4201	♂	30	.30	10[19] AM	34	10/24/86	996	4
4202	♂	28	.28		31		1000	0
4203	♂	27	.27		31		1000	0
4204	♂	29	.29		32		999	1
4205	♂	30	.30		32		1000	0
4206	♀	26	.26		28		1000	0
4207	♀	25	.25		28		999	1
4208	♀	29	.29		28		998	2
4209	♀	26	.26	↓	30		1000	0
4210	♀	27	.27	10[22] AM	28	10/24/86	1000	0
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	NM 10/24/86	NM 10/24/86	NM 11-21-86	NM 11-21-86

Observations: Immediate:

10/10 animals - no signs AHL 10-21-86

4 hr pd: 1/5 ♂ decreased body tone 4/5 ♂ 5/5 ♀ no signs NB 10/21/86

24 hr pd: 1/5 ♂ decreased body tone 4/5 ♂ 5/5 ♀ no signs NB 10/22/86

48 hr pd: 1/5 ♂ 1/5 ♀ decreased body tone 4/5 ♂ 4/5 ♀ no signs NB 10/23/86

72 hr pd 2/5 ♂ 1/5 ♀ decreased body tone 3/5 ♂ 4/5 ♀ no signs. NB 10/24/86

Dosed by: Nina Madison 10/21/86

Mean: 0.800 ± 1.316

t Values: 0.348

Nancy Gendreau 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

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NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH 309-SU-001-86

Sponsor: Sutton Laboratories

Test Article: Germall II

Description: White Powder

Date Initiated: 10-21-86

Date Terminated: 11-21-86

Dose Level: 2000 mg/kg @ 10 ml/kg → 200 mg/ml

DOSE: 2000 mg/kg SAMPLE TIME: 72 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4211	♂	30	.30	10:45 AM	32	10/24/86	999	1
4212	♂	28	.28	↓	33		998	2
4213	♂	29	.29		32		1000	0
4214	♂	31	.31		33		999	1
4215	♂	28	.28		31		1000	0
4216	♀	27	.27		28		999	1
4217	♀	26	.26		28		998	2
4218	♀	24	.24		27		999	1
4219	♀	27	.27	↓	29		999	1
4220	♀	25	.25	10:48 AM	27		999	1
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	NM 10/24/86	NM 10/24/86	NM 11-21-86	NM 11-21-86

Dosed by: Nina Malcom 10/21/86

Observations: Immediate:
1/5 ♂ 2/5 ♀ decreased body tone; no signs in remaining ♂ and ♀ animals
4 hrs pd: 1/5 ♂ 2/5 ♀ decreased body tone 4/5 ♂ 3/5 ♀ no signs NM 10/21/86
24 hrs pd: 1/5 ♂ 2/5 ♀ decreased body tone 4/5 ♂ 3/5 ♀ no signs NM 10/22/86
48 hrs pd: 3/5 ♂ 2/5 ♀ decreased body tone 2/5 ♂ 3/5 ♀ no signs NM 10/23/86
72 Hours pd 3/5 ♂ 2/5 ♀ decreased body tone 2/5 ♂ 3/5 ♀ no signs. NM 10/24/86

Mean: 1.000 ± 0.666

t Values: 0

Nancy Gengliewicz 11/21/86
Investigator Date

Ruth M. Eng 11-21-86
Study Director Date

NOTEBOOK # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

STUDY SHEET SINGLE DOSE

TITLE Cytogenetic Assay - Micronucleus Test STUDY NUMBER: PH309-SU-001-86

Sponsor: Sutton Laboratories Date Initiated: 10-21-86

Test Article: Germall II Date Terminated: 11-21-86

Description: White Powder Dose Level: 2800 mg/kg @ 10 ml/kg → 280 mg/ml

DOSE: 2800 mg/kg SAMPLE TIME: 72 Hours

Mouse No.	Sex	Initial Wt. (gm)	ml admin.	Time admin.	Final Wt. (gm)	Date of Sacrifice	No. of PCE	No. of PCE with Micronuclei
4221	♂	27	.27	11:20 AM	29		999	1
4222	♂	28	.28		Dead 27 10/21/86		—	—
4223	♂	30	.30		33	10/	1000	0
4224	♂	29	.29		32	24/	1000	0
4225	♂	30	.30		Dead 28 10/22/86		—	—
4226	♀	24	.24		26	86	1000	0
4227	♀	26	.26		28		999	1
4228	♀	24	.24		26		999	1
4229	♀	24	.24	↓	25		1000	0
4230	♀	26	.26	11:35 AM	27		1000	0
		AHL 10-21-86	AHL 10-21-86	AHL 10-21-86	NM 10/24/86	NM 10/24/86	NM 11-21-86	NM 11-21-86

Observations: Immediate:

3/5 ♀ 1/5 ♂ decreased body tone; no signs in remaining ♂♀ animals AHL 10-21-86

4 hrs post dose: 1/5 ♀ decreased body tone, abnormal gait 2/5 ♀ abnormal gait 3/5 ♀ [illegible] 10/22/86 2/5 ♀ no signs

2/5 ♂ decreased activity, abnormal gait, decreased body tone, tremors. 2/5 ♂ decreased body tone, abnormal gait 1/5 ♂ no signs NG 10/22/86

1/5 ♂ dead 7 hrs post dose #4222 ♂ Necropsy - Glandular section of stomach contained hemorrhagic areas, distended fluid filled (clear) Intestines distended, dark red in color, fluid filled. NM 10/21/86

24 Hr 10/22/86 #4225 ♂ Necropsy Stomach distended fluid filled (clear) Lung red in color NM 10/22/86 [illegible] red in color NM 10/22/86

Mean: 0.375 ± 0.517

t Values: 1.322

Dosed by: Nira Madison 10/21/86

Nancy Gongliewski 11/21/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

observations 24 hrs post dose 3/3 ♂ abnormal gait 1/3 ♂ decreased body tone 1 [illegible] 1/5 ♀ abnormal gait decreased body tone

(72 hrs p.d. 10/24/86) 2/1 ♂ decreased body tone 3/5 ♀ no signs

Page [illegible]

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-101-86

Sponsor: Sutton Laboratories, Inc

Values obtained from coded slides and subsequent decoding of test article Germall II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
1 10/29/86 NG	998	2	MC 72hr 4193♂
2	1000	0	2000 mg/Kg 72hr 4215♂
3	950	50	CP 30hr 4110♀
4	999	1	1200 mg/Kg 48hr 4165♂
5	999	1	2000 mg/Kg 48hr 4180♀
6	999	1	1200 mg/Kg 72hr 4207♀
7 10/29/86 NG	999	1	2000 mg/Kg 72hr 4218♀
8 10/30/86 NG	999	1	2800 mg/Kg 30hr 4141♂
9	995	5	1200 mg/Kg 30hr 4126♀
10	1000	0	2000 mg/Kg 48hr 4174♂
11	998	2	MC 30hr 4114♂
12	998	2	MC 48hr 4152♂
13	999	1	2800 mg/Kg 72hr 4227♀
14	1000	6	2000 mg/Kg 30hr 4132♂
15	1000	0	2000 mg/Kg 30hr 4133♂
16	999	1	MC 48hr 4156♀
17 10/30/86 NG	1000	0	1200 mg/Kg 48hr 4169♀

Nancy L Longfellow 10/30/86
Investigator Date

Kurt M. Sorg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article
Germall II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
18. 10/30/86 NG	976	24	CP 30hr 41020♂ / ~~1200 mg/kg 48hr 41169♀~~ IDE NG 11/20/8
19. 10/30/86 NG	997	3	2800 mg/kg 30hr 41479♀ / ~~CP 30hr 41080♂~~ IDE 11/20/8
20 10/31/86 NG	998	2	2800 mg/kg 48hr 41830♂ NG / ~~2800 mg/kg 30hr 41479♀~~ IDE
21.	1000	0	MC 48hr 41609♀ NG 11/20/8 / ~~2800 mg/kg 48hr 41830♂~~ IDE
22	1000	0	1200 mg/kg 48hr 41689♀ NG 11/20 / ~~MC 48hr 41607♀~~ IDE
23	1000	0	1200 mg/kg 72hr 42020♂ NG 11/20/8 / ~~1200 mg/kg 48hr 41689♀~~ IDE
24	973	27	CP 30hr 41040♂ NG 11/20/8 / ~~1200 mg/kg 72hr 42020♂~~ IDE
25.	999	1	2000 mg/kg 30hr 41389♀ / ~~CP 30hr 41040♂~~ IDE NG 11/20
26	1000	0	1200 mg/kg 72hr 42069♀ / ~~2000 mg/kg 30hr 41389♀~~ IDE NG 11/20
27.	997	3	2000 mg/kg 48hr 41779♀ / ~~1200 mg/kg 72hr 42069♀~~ IDE NG 11/20
28.	998	2	2000 mg/kg 30hr 41310♂
29.	999	1	2800 mg/kg 48hr 41899♀
30. ↓	997	3	2000 mg/kg 48hr 41750♂
31 10/31/86 NG	999	1	1200 mg/kg 30hr 41210♂
32. 11/3/86 NG	999	1	1200 mg/kg 30hr 41240♂
33. ↓	999	1	MC 30hr 41189♀
34. 11/3/86 NG	~~4~~ 1000 (IDE NG 11/3/86)	0	2000 mg/kg 48hr 41710♂

Nancy Gonzalez 11/3/86
Investigator / Date

Ruth M. Sorg 11-21-86
Study Director / Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article

Germell II

Code		Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode		
35	11/3/86 NS	1000	0	MC	48hr	41530♂
36		1000	0	1200 mg/Kg	72hr	42107♀
37		999	1	2800 mg/Kg	30hr	41487♀
38		999	1	1200 mg/Kg	30hr	41287♀
39		995	5	2800 mg/Kg	48hr	~~42079~~ 41887 IDE NG 11/20/86
40	↓	996	4	2000 mg/Kg	30hr	41320♂ IDE NG 11/20/86
41	11/3/86 NS	996	4	~~2800~~ IDE NG 11/20/86 MC	72hr	41948♂
42	11/4/86 NS	956	44	CP	30hr	41010♂
43		1000	0	2800 mg/Kg	30hr	41467♀
44		998	2	2000 mg/Kg	72hr	42120♂
45	↓	999	1	MC	30hr	41207♀
46	11/4/86 NS	997	3	1200 mg/Kg	48hr	41640♂
47	11/5/86 NS	997	3	2800 mg/Kg	48hr	41850♂
48		997	3	MC	30hr	41197♀
49		999	1	2000 mg/Kg	72hr	42110♂
50	↓	999	1	MC	72hr	42007♀
51	11/5/86 NS	1000	0	2800 mg/Kg	72hr	42307♀

Nancy Longlewicz 11/15/86
Investigator Date

Ruth M. Berg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc

Values obtained from coded slides and subsequent decoding of test article

Samuel II

Code		Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
52	11/5/86 NG	1000	0	2000 mg/Kg 48hr 41789
53		999	1	1200 mg/Kg 72hr 42040
54		999	1	2000 mg/Kg 48hr 41799
55	11/5/86 NG	999	1	MC 72hr 41967
56	11/6/86 NG	1000	0	MC 72hr 41979
57		999	1	1200 mg/Kg 48hr 41679
58		998	2	1200 mg/Kg 72hr 42089
59		999	1	2800 mg/Kg 30hr 41450
60		907	93	CP 30hr 41030
61		1000	0	2800 mg/Kg 72hr 42299
62		999	1	2000 mg/Kg 30hr 41399
63		1000	0	MC 72hr 41910
64		999	1	2000 mg/Kg 72hr 42209
65		999	1	1200 mg/Kg 48hr 41610
66		9993		2000 mg/Kg 48hr 41720
67		1000	0	1200 mg/Kg 72hr 42050
68	11/6/86	999	1	MC 48hr 41589

Nancy Gonglewski 11/6/86
Investigator Date

Ruth M. Sorg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-101-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article Germall II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
69 · 11/6/86 NY.	983	17	CP 30hr 4105 ♂
70 ·	999	1	1200 mg/Kg 48hr 4166 ♀
71 ·	1000	0	2800 mg/Kg 48hr 4181 ♂
72. 11/6/86 NY.	999	1	2000 mg/Kg 72hr 4214 ♂
73. 11/7/86 NY.	1000	0	2800 mg/Kg 72hr 4226 ♀
74	999	1	2800 mg/Kg 30hr 4149 ♀
75	1000	0	2800 mg/Kg 48hr 4190 ♀
76	1000	0	2000 mg/Kg 30hr 4140 ♀
77	1000	0	1200 mg/Kg 72hr 4209 ♀
78	999	1	2000 mg/Kg 72hr 4216 ♀
79	999	1	MC 30hr 4117 ♀
80	981	19	CP 30hr 4106 ♀
81	998	2	2800 mg/Kg 48hr 4186 ♀
82	996	4	1200 mg/Kg 72hr 4201 ♂
83 11/7/86 NY	1000	0	1200 mg/Kg 48hr 4170 ♀
84 11/10/86 NY	998	2	1200 mg/Kg 30hr 4129 ♀
85 11/10/86 NY	999	1	2000 mg/Kg 48hr 4173 ♂

Nancy Gonglewski 11/10/86
Investigator Date

Ruth N. Berg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-101-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article Gemfibrozil II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
86 11/10/86 NS	999	1	MC 30hr 41167
87 ↓	998	2	2000 mg/Kg 72hr 42177
88 11/10/86 NS	1000	0	2800 mg/Kg 72hr 42247
89 11/11/86 NS	9993	3	1200 mg/Kg 48hr 41637
90	1000 IDE NS 11/11/86	0	2800 mg/Kg 30hr 41507
91	999	1	2000 mg/Kg 30hr 41357
92	970	30	CP 30hr 41097
93	1000	0	2800 mg/Kg 48hr 41827
94	1000	0	MC 72hr 41957
95	1000	0	1200 mg/Kg 30hr 41257
96	1000	0	1200 mg/Kg 30hr 41227
97	1000	0	2800 mg/Kg 30hr 41427
98	998	2	MC 48hr 41557
99 ↓	998	2	MC 30hr 41137
100 11/11/86 NS	999	1	1200 mg/Kg 30hr 41237
101 11/12/86 NS	999	1	2800 mg/Kg 48hr 41877
102 11/12/86 NS	994	~~26~~ 26 IDE INC 11/12/86	CP 30hr 41077

Nancy Gongleuski 11/12/86
Investigator Date

Lucie H. Sorg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc

Values obtained from coded slides and subsequent decoding of test article Germall II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
103 11/12/86 NG	1000	0	1200 mg/Kg 30hr 41309
104	999	1	MC 48hr 41579
105	1000	0	2000 mg/Kg 72hr 42130
106	1000	0	2000 mg/Kg 30hr 41340
107 11/12/86 NG	998	2	2000 mg/Kg 30hr 41370
108 11/13/86 NG	1000	0	1200 mg/Kg 72hr 42030
109	999	1	MC 48hr 41540
110	999	1	2800 mg/Kg 72hr 42210
111	999	1	2000 mg/Kg 72hr 42199
112	1000	0	MC 72hr 41989
113	999	1	MC 72hr 41999
114	997	3	1200 mg/Kg 30hr 41279
115 11/13/86 NG	999	1	2000 mg/Kg 48hr 41769
116 11/14/86 NG	997	3	MC 30hr 41110
117	1000	0	MC 30hr 41120
118	998	2	1200 mg/Kg 48hr 41620
119 11/14/86 NG	999	1	2800 mg/Kg 72hr 42289

Nancy Gongliewski 11/14/86
Investigator /Date

Ruth M. Sarg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article Germall II

Code	Polychromatic Erythrocytes	Polychromatic Erythrocytes with Micronuclei	Decode
120 · 11/14/86 NG	999	1	MC 72hr 4193♂
121	1000	0	MC ~~48~~ 30hr 41~~54~~ 15♂ 1DE NG 11/20/86
122	1000	0	2800 mg/Kg 72hr 4223♂
123	998	2	2800 mg/Kg 30hr 4144♂
124	976	24	CP 30hr 4108♀
125	997	3	MC 48hr 4151♂
126	1000	0	MC 48hr 4159♀
127 · 11/14/86 NG	998	2	2800 mg/Kg 30hr 4143♂

Nancy Gongliewski 11/14/86
Investigator Date

Kurt M. Sorg 11-21-86
Study Director Date

CHTRMS(10)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SV-001-86

Sponsor: Sutton Laboratories, Inc.

Values obtained from coded slides and subsequent decoding of test article Germall II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code		Mature Micronucleated Erythrocytes
1. 10/29/86 NS	I	1
2.		0
3	III	3
4.		0
5		0
6		0
7 10/29/86 NS	I	1
8 10/30/86 NS	I	1
9		0
10	I	1
11	II	2
12		0
13		0
14		0
15		0
16		0
17 10/30/86 NS		0

Nancy Gongleuski 10/30/86
Investigator Date

Rusty N. Sorg 11-21-86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Bernell II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code	Mature Micronucleated Erythrocytes
18. 10/30/86 NS	1
19. 10/30/86 NS	0
20 10/31/86 NS	0
21	0
22	0
23	0
24	0
25	0
26	1
27	0
28	2
29	0
30	1
31 10/31/86 NS	0
32 11/3/86 NS	0
33	0
34 11/3/86 NS	0

Nancy Englewood 11/3/86
Investigator Date

Ruth M. Sag 11-4-86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemnell II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code		Mature Micronucleated Erythrocytes
35 11/3/86 NS		0
36		0
37		0
38		0
39		0
40	1	1
41 11/3/86 NS		6
42 11/4/86	111	3
43		0
44		0
45	1	1
46 11/4/86 NS	1	1
47 11/5/86 NS		0
48		0
49		0
50		0
51 11/5/86 NS		0

Nancy L Longkowski 11/5/86
Investigator Date

Ruth M. Sag 11-21-86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemnell II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code		Mature Micronucleated Erythrocytes
52	11/5/86 NB	0
53	↓	0
54	11/5/86 NB	0
55	11/6/86 NB	0
56		0
57		0
58	1	1
59		0
60	11	2
61	1	1
62		0
63		0
64		0
65		0
66		0
67	↓	0
68	11/6/86 NB 11	2

Nancy Longhenrik 11/6/86
Investigator / Date

Lisa H. Sarg 11/21/86
Study Director / Date

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CHTRMS(11)

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PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Lemuell II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code	Mature Micronucleated Erythrocytes
69 11/6/86 NG	2
70	0
71	6
72 11/6/86 NG	0
73 11/7/86	6
74	1
75	0
76	0
77	0
78	0
79	1
80	3
81	0
82	0
83 11/7/86 NG	0
84 11/10/86 NG	0
85 11/10/86 NG	0

Nancy Gongleewski 11/10/86
Investigator Date

Ruth M. Sorg 11/21/86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Germall II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code		Mature Micronucleated Erythrocytes
86	11/10/86 NS	2
87	↓	0
88	11/10/86 NS	0
89	11/11/86 NS	2
890		0
10 ENG 91		0
92		1
93		0
94		0
95		0
96		0
97		1
98		0
99	↓	0
100	11/11/86 NS	0
101	11/12/86 NS	0
102	11/12/86 NS	0

Nancy Gongliwnok 11/12/86
Investigator Date

Ruth M. Sey 11-24-86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Bernadette

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code	Mature Micronucleated Erythrocytes
103 11/12/86 NG	0
104	0
105	1
106	0
107 11/12/86 NG II	2
108 11/13/86 NG	0
109	0
110	0
111	0
112	0
113	0
114	0
115 11/13/86 NG	0
116 11/14/86 NG	0
117	0
118	1
119 11/14/86 NG	0

Nancy Gonglewski 11/14/86
Investigator Date

Curt M. Sorg 11/21/86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemnell II

# of Mature Micronucleated Erythrocytes seen in the fields scanned to obtain 1000 PCEs.

Code		Mature Micronucleated Erythrocytes
120	11/14/86 NB 1	1
121.		0
122.		0
123.		0
124.	11	2
125.	1	1
126.	1	1
127.	11/14/86 NB 1	1

Nancy Gonglewski 11/14/86
Investigator Date

Ruth M. Seng 11/24/86
Study Director Date

CHTRMS(11)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Carmell II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes	Decode
1 Nm 10/31/86	662	338	MC 72 Hrs. 4193 ♂
2	601	399	2000 mg/kg 72 Hr 4215 ♂
3	538	462	CP 30 Hrs 4110 ♀
4	682	318	1200 mg/kg 48 Hrs 4165 ♂
5	598	402	2000 mg/kg 48 Hrs 4180 ♀
6	586	414	1200 mg/kg 72 Hrs 4207 ♀
7	595	405	2000 mg/kg 72 Hrs 4218 ♀
8	479	521	2800 mg/kg 30 Hrs 4141 ♂
9	722	278	1200 mg/kg 30 Hrs 4126 ♀
10	585	415	2000 mg/kg 48 Hrs 4174 ♂
11	489	511	MC 30 Hrs 4114 ♂
12	589	411	MC 48 Hrs 4152 ♂
13. ↓	734	266	2800 mg/kg 72 Hrs 4227 ♀
14 Nm 10/31/86	496	504	2000 mg/kg 30 Hrs 4132 ♂
15. Nm 11/5/86	627	373	2000 mg/kg 30 Hrs 4133 ♂
16 ↓	560	440	MC 48 Hrs 4156 ♀
17 Nm 11/5/86	665	335	1200 mg/kg 48 Hrs 4169 ♀

Nilesh Madison 11/5/86
Investigator Date

Robert M. Sorg 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. 44309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemnell II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes	Decode		
18 KM 11/5/86	465	535	CP	30 Hrs	4102 ♂
19	560	440	2800 mg/kg	30 Hrs	4147 ♀
20 KM 11/5/86	656	344	2800 mg/kg	48 Hrs	4183 ♂
21 KM 11/6/86	610	390	MC	48 Hrs	4160 ♀
22	623	377	1200 mg/kg	48 Hrs	4168 ♀
23	554	446	1200 mg/kg	72 Hrs	4202 ♂
24	402	598	CP	30 Hrs	4104 ♂
25	650	350	2000 mg/kg	30 Hr	4138 ♀
26	594	406	1200 mg/kg	72 Hr	4206 ♀
27	676	324	2000 mg/kg	48 Hr	4177 ♀
28	453	547	2000 mg/kg	30 Hrs	4131 ♂
29	677	323	2800 mg/kg	48 Hrs	4189 ♀
30	540	460	2000 mg/kg	48 Hrs	4175 ♂
31	515	487	1200 mg/kg	30 Hrs	4121 ♂
32	502	498	1200 mg/kg	30 Hrs	4124 ♂
33	540	460	MC	30 Hrs	4118 ♀
34 KM 11/6/86	476	524	2000 mg/kg	48 Hrs	4171 ♂

Vera Madison 11/6/86
Investigator Date

Ruth M. Sorg 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Germall II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes		Decode
35 NM 11/6/86	621	379	MC	48 Hrs 4153 ♂
36	653	347	1200 mg/kg	72 Hrs 4210 ♀
37	670	330	2800 mg/kg	30 Hrs 4148 ♀
38	739	261	1200 mg/kg	30 Hrs 4128 ♀
39	582	418	2800 mg/kg	48 Hrs 4188 ♀
40	682	318	2000 mg/kg	30 Hrs 4136 ♀
41	517	483	MC	72 Hrs 4194 ♂
42 NM 11/6/86	496	504	CP	30 Hrs 4101 ♂
43 NM 11/7/86	709	291	2800 mg/kg	30 Hrs 4146 ♀
44	575	425	2000 mg/kg	72 Hrs 4212 ♂
45	631	369	MC	30 Hrs 4120 ♀
46	677	323	1200 mg/kg	48 Hrs 4164 ♂
47	601	399	2800 mg/kg	48 Hrs 4185 ♂
48	597	403	MC	30 Hrs 4119 ♀
49	639	361	2000 mg/kg	72 Hrs 4211 ♂
50	588	412	MC	72 Hrs 4200 ♀
51 NM 11/7/86	701	299	2800 mg/kg	72 Hrs 4230 ♀

Investigator 11/7/86 Date

Study Director 11/20/86 Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemnell II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes	Decode
52 RM 11/7/86	591	409	2000 mg/kg 48 Hrs 4178 ♀
53	513	487	1200 mg/kg 72 Hrs 4204 ♂
54	577	423	2000 mg/kg 48 Hr 4179 ♀
55	634	366	MC 72 Hrs 4196 ♀
56	635	365	MC 72 Hrs 4197 ♀
57	642	358	1200 mg/kg 48 Hrs 4167 ♀
58	511	489	1200 mg/kg 72 Hrs 4208 ♀
59 RM 11/7/86	584	416	2500 mg/kg 30 Hrs 4145 ♂
60 RM 11/10/86	388	612	CP 36 Hrs 4103 ♂
61	697	303	2500 mg/kg 72 Hrs 4229 ♀
62	586	414	2000 mg/kg 30 Hrs 4139 ♀
63	564	436	MC 72 Hrs 4191 ♂
64	596	404	2000 mg/kg 72 Hrs 4220 ♀
65	558	442	1200 mg/kg 48 Hrs 4161 ♂
66	530	470	2000 mg/kg 48 Hrs 4172 ♂
67	558	442	1200 mg/kg 72 Hrs 4205 ♂
68 RM 11/10/86	609	391	MC 48 Hrs 4158 ♀

Investigator 11/10/86 Date

Study Director 11/20/86 Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. 44309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Bernell II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes		Decode	
69 RW 11/10/86	376	624	CP	30 Hrs	4105 ♂
70	576	424	1200 mg/kg	48 Hrs	4166 ♀
71	611	389	2800 mg/kg	48 Hrs	4181 ♂
72	538	462	2000 mg/kg	72 Hrs	4214 ♂
73	701	299	2800 mg/kg	72 Hrs	4226 ♀
74	619	381	2800 mg/kg	30 Hrs	4149 ♀
75	610	390 ~~390~~ 10c 11/10/86	2800 mg/kg	48 Hrs	4190 ♀
76	706	294	2000 mg/kg	30 Hrs	4140 ♀
77 RW 11/10/86	556	444	1200 mg/kg	72 Hrs	4209 ♀
78 RW 11/11/86	497	503	2000 mg/kg	72 Hrs	4216 ♀
79	653	347	MC	30 Hrs	4117 ♀
80	542	458	CP	30 Hrs	4106 ♀
81	620	380	2800 mg/kg	48 Hrs	4186 ♀
82	583	417	1200 mg/kg	72 Hrs	4201 ♂
83	662	338	1200 mg/kg	48 Hrs	4170 ♀
84	539	461	1200 mg/kg	30 Hrs	4129 ♀
85 RW 11/11/86	586	414	2000 mg/kg	48 Hrs	4173 ♂

Nina Madison 11/11/86
Investigator Date

Ruth M. Song 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. 44309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article

Kermelott

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes	Decode
86 KM 11/11/86	575	425	MC 30 Hrs 4116♀
87 KM 11/11/86	611	389	2000 mg/kg 72 Hrs 4217♀
88 KM 11/12/86	552	448	2800 mg/kg 72 Hrs 4224♂
89	600	400	1200 mg/kg 48 Hrs 4163♂
90	572	428	2800 mg/kg 30 Hrs 4150♀
91	617	383	2000 mg/kg 30 Hrs 4135♂
92	529	471	CP 30 Hrs 4109♀
93	534	466	2800 mg/kg 48 Hrs 4182♂
94	487	513	MC 72 Hrs 4195♂
95	351	649	1200 mg/kg 30 Hrs 4125♂
96	557	443	1200 mg/kg 30 Hrs 4122♂
97	502	498	2800 mg/kg 30 Hrs 4142♂
98	678	322	MC 48 Hrs 4155♂
99	390	610	MC 30 Hrs 4113♂
100	562	438	1200 mg/kg 30 Hrs 4123♂
101	636	364	2800 mg/kg 48 Hrs 4187♀
102 KM 11/12/86	532	468	CP 30 Hrs 4107♀

Nina Madsen 11/12/86
Investigator Date

Mark H. Sorg 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. PH309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Gemmell II

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes	Decode
103 RM 11/12/86	644	356	1200 mg/kg 30 Hrs 4130 ♀
104 ↓	544	456	MC 48 Hrs 4157 ♀
105 RM 11/12/86	456	544	2000 mg/kg 72 Hrs 4213 ♂
106 RM 11/18/86	608	392	2000 mg/kg 30 Hrs 4134 ♂
107	564	436	2000 mg/kg 30 Hrs 4137 ♀
108	564	436	1200 mg/kg 72 Hrs 4203 ♂
109	646	360	MC 48 Hrs 4154 ♂
110	553	447	2800 mg/kg 72 Hrs 4221 ♂
111	655	345	2000 mg/kg 72 Hrs 4219 ♀
112	410	590	MC 72 Hrs 4198 ♀
113	541	459	MC 72 Hrs 4199 ♀
114	696	304	1200 mg/kg 30 Hrs 4127 ♀
115	608	392	2000 mg/kg 48 Hrs 4176 ♀
116	300	700	MC 30 Hrs 4111 ♂
117	595	405	MC 30 Hrs 4112 ♂
118 ↓	652	348	1200 mg/kg 48 Hrs 4162 ♂
119 RM 11/18/86	699	301	2800 mg/kg 72 Hrs 4228 ♀

Mike Madison 11/18/86
Investigator Date

Ruth M. Sorg 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

SCORING SHEET

PHARMAKON RESEARCH INTERNATIONAL, INC.

TITLE: Cytogenetic Assay - Micronucleus Test STUDY NO. 84309-SU-001-86

Sponsor: Sutton Laboratories

Values obtained from coded slides and subsequent decoding of test article Vehicle

Code	Polychromatic Erythrocytes	Normochromatic Erythrocytes		Decode	
120 KM 11/19/86	477	523	MC	72 Hrs	4192
121	646	354	MC	30 48 Hrs Toe KM 11/19/86	4115
122	477	523	2800 mg/kg	72 Hrs	42230
123	579	421	2800 mg/kg	30 Hrs	4144
124	467	533	CP	30 Hrs	4108
125	552	448	MC	48 Hrs	4151
126	692	308	MC	48 Hrs	41597
127 KM 11/19/86	550	450	2800 mg/kg	30 Hrs	41430

Nicki Madison 11/19/86
Investigator Date

Keith M. Sorg 11/20/86
Study Director Date

CHTRMS (9)

Notebook # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

CODE SHEET

Sponsor: Sutton Laboratories
PH 309-SU-001-86
Test Article: Germall II
Dose Level: 1200, 2000 and 2800 mg/kg

Coded by: Donna K. Hastko
Date 10/29/86
Decoded by: [illegible]
Date 11-19-86

Code	Dose	Harvest Time	Animal #	Code	Dose	Harvest Time	Animal #
1	MC	72 HR.	4193 ♂	27	2000 mg/Kg	48 HR	4177 ♀
2	2000 mg/Kg	72 HR.	4215 ♂	28	2000 mg/Kg	30 HR	4131 ♂
3	CP	30 HR	4110 ♀	29	2800 mg/Kg	48 HR	4189 ♀
4	1200 mg/Kg	48 HR	4165 ♂	30	2000 mg/Kg	48 HR	4175 ♂
5	2000 mg/Kg	48 HR	4180 ♀	31	1200 mg/Kg	30 HR	4121 ♂
6	1200 mg/Kg	72 HR	4207 ♀	32	1200 mg/Kg	30 HR	4124 ♂
7	2000 mg/Kg	72 HR	4218 ♀	33	MC	30 HR	4118 ♀
8	2800 mg/Kg	30 HR	4141 ♂	34	2000 mg/Kg	48 HR	4171 ♂
9	1200 mg/Kg	30 HR	4126 ♀	35	MC	48 HR	4153 ♂
10	2000 mg/Kg	48 HR	4174 ♂	36	1200 mg/Kg	72 HR	4210 ♀
11	MC	30 HR	4114 ♂	37	2800 mg/Kg	30 HR	4148 ♀
12	MC	48 HR	4152 ♂	38	1200 mg/Kg	30 HR	4128 ♀
13	2800 mg/Kg	72 HR	4227 ♀	39	2800 mg/Kg	48 HR	4188 ♀
14	2000 mg/Kg	30 HR	4132 ♂	40	2000 mg/Kg	30 HR	4136 ♀
15	2000 mg/Kg	30 HR	4133 ♂	41	MC	72 HR	4194 ♂
16	MC	48 HR	4156 ♀	42	CP	30 HR	4101 ♂
17	1200 mg/Kg	48 HR	4169 ♀	43	2800 mg/Kg	30 HR	4146 ♀
18	CP	30 HR	4102 ♂	44	2000 mg/Kg	72 HR	4212 ♂
19	2800 mg/Kg	30 HR	4147 ♀	45	MC	30 HR	4120 ♀
80	2800 mg/Kg	48 HR	4183 ♂	46	1200 mg/Kg	48 HR	4164 ♂
21	MC	48 HR	4160 ♀	47	2800 mg/Kg	48 HR	4185 ♂
22	1200 mg/Kg	48 HR	4168 ♀	48	MC	30 HR	4119 ♀
23	1200 mg/Kg	72 HR	4202 ♂	49	2000 mg/Kg	72 HR	4211 ♂
24	CP	30 HR	4104 ♂	50	MC	72 HR	4200 ♀
25	2000 mg/Kg	30 HR	4138 ♀	51	2800 mg/Kg	72 HR	4230 ♀
26	1200 mg/Kg	72 HR	4206 ♀	52	2000 mg/Kg	48 HR	4178 ♀

Study Director Ruth M. Sorg 11-21-86

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CHTRMS(12)

Notebook # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

CODE SHEET

Sponsor: Sutton Laboratories
PR 309-SU-001-86
Test Article: Gemcell II
Dose Level: 1200, 2000 and 2800 mg/kg

Coded by: [illegible] 10/29/86
Date [illegible]
Decoded by: [illegible]
Date 11-19-86

Code	Dose	Harvest Time	Animal #	Code	Dose	Harvest Time	Animal #
53	1200 mg/Kg	72 HR	4204 ♂	79	MC	30 HR	4111 ♀
54	2000 mg/Kg	48 HR	4179 ♀	80	CP	30 HR	4106 ♀
55	MC	72 HR	4196 ♀	81	2800 mg/Kg	48 HR	4186 ♀
56	MC	72 HR	4197 ♀	82	1200 mg/Kg	72 HR	4201 ♂
57	1200 mg/Kg	48 HR	4167 ♀	83	1200 mg/Kg	48 HR	4170 ♀
58	1200 mg/Kg	72 HR	4208 ♀	84	1200 mg/Kg	30 HR	4129 ♀
59	2800 mg/Kg	30 HR	4145 ♂	85	2000 mg/Kg	48 HR	4173 ♂
60	CP	30 HR	4103 ♂	86	MC	30 HR	4116 ♀
61	2800 mg/Kg	72 HR	4229 ♀	87	2000 mg/Kg	72 HR	4217 ♀
62	2000 mg/Kg	30 HR	4139 ♀	88	2800 mg/Kg	72 HR	4224 ♂
63	MC	72 HR	4191 ♂	89	1200 mg/Kg	48 HR	4163 ♂
64	2000 mg/Kg	72 HR	4220 ♀	90	2800 mg/Kg	30 HR	4150 ♀
65	1200 mg/Kg	48 HR	4161 ♂	91	2000 mg/Kg	30 HR	4135 ♂
66	2000 mg/Kg	48 HR	4172 ♂	92	CP	30 HR	4109 ♀
67	1200 mg/Kg	72 HR	4205 ♂	93	2800 mg/Kg	48 HR	4182 ♂
68	MC	48 HR	4158 ♀	94	MC	72 HR	4195 ♂
69	CP	30 HR	4105 ♂	95	1200 mg/Kg	30 HR	4125 ♂
70	1200 mg/Kg	48 HR	4166 ♀	96	1200 mg/Kg	30 HR	4122 ♂
71	2800 mg/Kg	48 HR	4181 ♂	97	2800 mg/Kg	30 HR	4142 ♂
72	2000 mg/Kg	72 HR	4214 ♂	98	MC	48 HR	4155 ♂
73	2800 mg/Kg	72 HR	4226 ♀	99	MC	30 HR	4113 ♂
74	2800 mg/Kg	30 HR	4149 ♀	100	1200 mg/Kg	30 HR	4123 ♂
75	2800 mg/Kg	48 HR	4190 ♀	101	2800 mg/Kg	48 HR	4187 ♀
76	2000 mg/Kg	30 HR	4140 ♀	102	CP	30 HR	4107 ♀
77	1200 mg/Kg	72 HR	4209 ♀	103	1200 mg/Kg	30 HR	4130 ♀
78	2000 mg/Kg	72 HR	4216 ♀	104	MC	48 HR	4157 ♀

Study Director Ruth M. Sorg 11-21-86

CHTRMS(12)

Notebook # 1123

PHARMAKON RESEARCH INTERNATIONAL, INC.

CODE SHEET

Sponsor: Sutton Laboratories
PH 309-SU-001-86
Test Article: Germall II
Dose Level: 1200, 2000 and 2800 mg/kg

Coded by: Donna Hrastko 10/29/86
Date ~~10/30/86~~ IDE DH 12/5/86 10/29/86
Decoded by: [illegible]
Date 11-19-86

Code	Dose	Harvest Time	Animal #	Code	Dose	Harvest Time	Animal #
105	2000 mg/Kg	72 HR	4213 ♂				
106	2000 mg/Kg	30 HR	4134 ♂				
107	2000 mg/Kg	30 HR	4137 ♀				
108	1200 mg/Kg	72 HR	4203 ♂				
109	MC	48 HR	4154 ♂				
110	2800 mg/Kg	72 HR	4221 ♂				
111	2000 mg/Kg	72 HR	4219 ♀				
112	MC	72 HR	4198 ♀				
113	MC	72 HR	4199 ♀				
114	1200 mg/Kg	30 HR	4127 ♀				
115	2000 mg/Kg	48 HR	4176 ♀				
116	MC	30 HR	4111 ♂				
117	MC	30 HR	4112 ♂				
118	1200 mg/Kg	48 HR	4162 ♂				
119	2800 mg/Kg	72 HR	4228 ♀				
120	MC	72 HR	4192 ♂				
121	MC	30 ~~48~~ HR (72 [illegible] 11-20-86)	~~4157~~ 4155 ♂				
122	2800 mg/Kg	72 HR	4223 ♂				
123	2800 mg/Kg	30 HR	4144 ♂				
124	CP	30 HR	4108 ♀				
125	MC	48 HR	4151 ♂				
126	MC	48 HR	4159 ♀				
127	2800 mg/Kg	30 HR	4143 ♂				

Study Director Ruth M. Lorg 11-21-86

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CHTRMS(12)

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

TEST ARTICLE AT0214

(Germall II -- Diazolidinyl Urea)

MICROBIOLOGICAL ASSOCIATES

Microbiological Associates
A Unit of Whittaker Corporation
5221 River Road
Bethesda, Maryland 20816
(301) 654-3400
Telex No. 90-8793

Whittaker

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

Sponsor:

Testing Facility: 1530 East Jefferson Street
Rockville, Maryland 20852

Study No.: T2039.501

Test Article I.D.: AT0214

Test Article Lot No.: None Provided

Test Article Description: White Powder

Storage Conditions: Room Temperature with Desiccation, Protected from Light

Date Received: 6/7/83

Date Study Started: 6/30/83

Date Study Completed: 7/28/83

Report Date: 7/29/83

Study Coordinator:

Study Director: Steve R. Haworth, Ph.D.
Microbiological Associates

Steve R. Haworth 7/28/83
Steve R. Haworth, Ph.D. Date
Study Director

Timothy E. Lawlor 7/28/83
Timothy E. Lawlor Date
Alternate Study Director

Jeanene K. Burke 7/28/83
Jeanene K. Burke Date
Group Leader

Sheila M. Olewine 7/25/83
Sheila M. Olewine Date
Biologist

Robin J. Plunkett 7/28/83
Robin J. Plunkett Date
Biologist

Linda M. Coyle 7/28/83
Linda M. Coyle Date
Biologist

QUALITY ASSURANCE STATEMENT

Study Title: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

Study Number: T2039.501

Study Director: S. Haworth, Ph.D.

Initiation Date: June 30, 1983

Review Completed Date: July 29, 1983

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc. are examined in order to assure that the study is performed in accordance with the Good Laboratory Practices regulations and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

DATE OF INSPECTION	PHASE INSPECTED	REPORT SUBMITTED TO STUDY DIRECTOR	REPORT SUBMITTED TO MANAGEMENT
6/15/83	Protocol review	6/15/83	6/15/83
6/30/83	Initial toxicity: Strain characterization	6/30/83	7/1/83
	Treatment & plating of the cultures		
7/28/83	Final report	7/28/83	7/29/83

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Nona S. Karten 7/29/83

Nona S. Karten Date
Associate Director RA/QA

Introduction

test article AT0214 (MA #T2039) was received on June 7, 1983, for testing in the Salmonella/mammalian-microsome mutagenicity assay using five tester strains, TA98, TA100, TA1535, TA1537 and TA1538, both with and without metabolic activation by Aroclor induced rat liver microsomes.

Conclusions

The results of the Salmonella/mammalian-microsome mutagenicity assay indicate that under the conditions of this study, test article AT0214 (MA #T2039) did not cause a positive response on any of the tester strains with or without metabolic activation by Aroclor induced rat liver microsomes.

It should be noted that in the presence of rat liver microsomes, there was a two to three-fold non-dose-responsive increase in TA1537 revertants per plate. There were also less than two-fold increases in TA98 and TA97 revertants per plate observed in the same experiments. However, none of the increases met the criteria for a positive response as described in the protocol used for this study.

MATERIALS AND METHODS[1]

Media Preparation

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 8 g/liter Difco Bacto Agar and 5 g/liter NaCl. After autoclaving, the molten agar was distributed in 100 ml aliquots into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 ml/100 ml agar of a sterile solution which contained 0.5 mM L-histidine and 0.5 mM D-biotin. Twenty-five ml of sterile deionized water was added per 100 ml top agar when it was used in assays without metabolic activation. This ensured that final top agar and amino acid supplement concentrations were the same on plates with or without metabolic activation.

Top Agar for Viable Count Determination: Minimal top agar as described above was supplemented with 35 ml/100 ml agar of a sterile solution which contained 1.4 mM L-histidine and 0.12 mM D-biotin.

Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E.[2]

Nutrient Broth: Nutrient broth used for growing overnight cultures of the tester strains contained 25 g per liter of Nutrient Broth No. 2 (Oxoid).

Nutrient Bottom Agar: Nutrient bottom agar was Vogel-Bonner[3] minimal medium E supplemented with 25 g per liter of Nutrient Broth No. 2 (Oxoid).

---

[1]The experimental materials, methods and procedures are based on those described by Ames, B. N., et al. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31: 347-364, 1975.

[2]Vogel, H. J. and D. M. Bonner, Acetylornithinase of E. coli: partial purification and some properties, J. Biol. Chem., 218:97-106 (1956).

[3]Ibid.

Tester Strain Diluent: Diluent for tester strain titering contained Vogel-Bonner salt solution[4] supplemented with 10% Nutrient Broth.

Test Article Diluent: The solvent used for diluting the test article was deionized, distilled H_2O.

Tester Strains

The tester strains used were the histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as suggested by Ames.[5]

GENOTYPE OF THE TA STRAINS USED FOR MUTAGEN TESTING

Histidine mutation			Additional mutations		
hisG46	hisC3076	hisD3052	LPS	Repair	R factor
TA1535	TA1537	TA1538	rfa	uvrB	-
TA100		TA98	rfa	uvrB	+R

The tester strains possess characteristics which greatly enhance their sensitivity to mutagenic materials.

Each strain possesses the rfa wall mutation which has resulted in the loss of much of the lipopolysaccharide layer that coats the surface of the bacteria. This allows the entry into the bacterial cells of large ring compounds that would otherwise be excluded by a normal intact cell wall. Secondly, a stable mutation resulting in the loss of an excision repair system (uvrB) further enhances each tester strain's sensitivity to some mutagens. Finally, strains TA98 and TA100 contain the pkM101 plasmid which further increases the sensitivity of these two strains to some mutagens.

---

[4]Vogel, H. J., et al., op cit.

[5]Ames, B. N., et al., op cit.

TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frame shift mutagens. TA100 and TA1535 are reverted by mutagens that cause base substitutions.

Tester strains in use at Microbiological Associates were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Tester strain stocks were stored in liquid nitrogen, and fresh cultures were inoculated directly from these frozen stocks. Broth cultures were grown overnight at 37°C with shaking. At the time of its use in the mutagenicity assay, each culture was checked, as described by Ames, for the presence of the rfa wall mutation and strains TA98 and TA100 were checked for the presence of the pkM101 plasmid.[6]

Toxicity Determination and Selection of Maximum Test Article Dose Level

The test article was checked for toxicity to the tester strains up to a concentration of 10 mg/plate. An aliquot from ten dilutions of the test article was plated with an overnight TA100 culture on selective minimal agar, both in the presence and absence of metabolic activation. Toxicity is detectable by a decrease in the number of revertant colonies occurring per plate and/or by a thinning or disappearance of the background bacterial lawn. The highest concentration of test article used in the subsequent mutagenicity assay was that which gave a detectable reduction in the number of revertants per plate and/or produced a thinning or disappearance of the background bacterial lawn.

When necessary, separate dose levels are plated for the portion of the assay with metabolic activation and the without metabolic activation portion of the assay.

The results of the preliminary toxicity determination are presented in the Results section of the final report.

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[6]Ames, B. N., et al., op cit.

Plating Procedures for the Mutagenicity Assay

Test System Identification: Each plate was labeled using indelible ink with a code system which identifies the test article, test phase, dose level and activation as described in detail in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

Test Article: The test article was solubilized and serially diluted immediately before its use in the mutagenicity assay. Five doses of the test article were plated with all five tester strains (TA98, TA100, TA1535, TA1537, TA1538) with metabolic activation and without metabolic activation. All positive controls, solvent controls and test article doses were plated in triplicate. Without metabolic activation, 50 µl of tester strain and 50 µl of solvent or test article were added to 2.5 ml of molten selective top agar at 45°C. With metabolic activation, 50 µl of tester strain, 50 µl of solvent or test article, and 0.5 ml of S-9 mix were added to 2.0 ml of molten selective top agar at 45°C. After vortexing, the mixture was overlayed onto the surface of 25 ml of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37°C.

Positive Controls: All combinations of positive controls and tester strains plated along with the assay are listed below:

Strain	Activation	Positive Controls	Conc. per Plate
TA98	+	2-Aminoanthracene	4.0 µg
TA98	-	2-Nitrofluorene	5.0 µg
TA100	+	2-Aminoanthracene	4.0 µg
TA100	-	Sodium Azide	5.0 µg
TA1535	+	2-Aminoanthracene	4.0 µg
TA1535	-	Sodium Azide	5.0 µg
TA1537	+	2-Aminoanthracene	4.0 µg
TA1537	-	9-Aminoacridine	75 µg
TA1538	+	2-Aminoanthracene	4.0 µg
TA1538	-	2-Nitrofluorene	5.0 µg

Source and Grade

9-Aminoacridine (CAS #90-45-9), Sigma Chemical Co., grade II, ~90% pure

2-Aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade

2-Nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98% pure

Sodium Azide (CAS #26628-22-8), Sigma Chemical Co., practical grade

Tester Strain Titers: Tester strain titers were determined by viable count assays on nutrient agar plates. The averaged number of cells plated per plate are reported on the individual strain data forms.

Test Article Sterility Determination: The most concentrated test article dilution for the mutagenicity assay was checked for sterility by plating a 50 µl aliquot of the dilution on selective agar.

Liver Microsomal Enzymes

Preparation of S-9 Homogenate: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 at 500 mg/kg. The Aroclor was diluted in corn oil to a concentration of 200 mg/ml. Five days after their i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised. The rats were fasted for 12 hours immediately preceding sacrifice.

The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 0-4°C. The liver from each rat was excised and placed in approximately 20 ml of 0.15M KCl contained in a pre-weighed beaker. After weighing the liver, it was transferred to another beaker containing 3 volumes of 0.15M KCl (3 ml/g of wet liver) where it was minced with sterile scissors. The minced liver was homogenized in a Potter-Elvehjen apparatus with a teflon pestle. The homogenate was centrifuged

at 9000 x g for 10 minutes in the SS-34 rotor of a Sorvall SS-3 centrifuge. The supernatant (referred to by Ames as the S-9 fraction) was decanted, and small aliquots were distributed into freezing ampules which were stored at $\leq -70^{\circ}C$.

Preparation of S-9 Mix: The S-9 mix was prepared immediately before its use in the mutagenicity assay.

The microsomal enzyme reaction mixture (S-9 mix) which was added to the soft agar overlay contained the following components per ml:

S-9	0.10 ml
0.2M $MgCl_2$/.825M KCl	0.04 ml
0.04M NADP	0.10 ml
0.04M Glucose-6-phosphate	0.10 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
H_2O	0.56 ml
	1.00 ml

Colony Counting

Revertant colonies for a given tester strain within a given test article dilution series were counted either entirely by automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting were counted manually.

The condition of the background bacterial lawn was evaluated for evidence of test article toxicity, by using a dissecting microscope. This toxicity was scored relative to the solvent control plate and recorded along with the revertant count for that plate on the individual strain data forms using the code system on page 14.

Analysis of Data

All platings were done in triplicate. For each triplicate plating, an average and standard deviation were calculated. The calculations were done on a Hewlett-Packard HP·25 programmable calculator which employs the following equations:

Average ($\bar{x}$)

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

Standard Deviation (S_x)

$$S_x = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

Evaluation of Mutagenicity Assay Data

For a test article to be considered positive, it must cause at least a doubling in the mean revertants per plate of at least one tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article. In those cases where the observed dose-responsive increase in TA1537 or TA1538 revertants per plate is less than three-fold, the response must be reproducible.

Archives

All experimental records of the study are maintained in the Microbiological Associates' archives located at 1530 East Jefferson Street, Rockville, Maryland, 20852.

Stability of the Test Article

The stability of the test article under the actual experimental conditions used in this study was not determined by Microbiological Associates.

RESULTS

The Salmonella/Mammalian-Microsome Mutagenicity Assay is divided into two phases. The first phase, the preliminary toxicity determination, is used to establish the dose range over which the test article will be assayed. The second phase is the mutagenicity assay of the test article.

Results of the preliminary toxicity determination of test article AT0214 (MA #T2039) are presented in Tables 1 and 2.

PRELIMINARY TOXICITY DETERMINATION

OF TEST ARTICLE

T2039-A1
Experiment I.D.

T2039.501
Study Number

Table 1

AT0214
Test Article Identification

With S-9 Activation

Test Article Concentration µg/Plate	TA100 Revertants/ Plate	TA100 Background Bacterial Lawn*
H_2O 50 µl	84	1
0.0001	70	1
0.001	90	1
0.010	94	1
0.033	84	1
0.10	85	1
0.33	80	1
1.0	81	1
10	75	1
100	80	1
1,000	41	4

*Refer to background bacterial lawn evaluation code key

Date Plated 6/30/83

Dilutions of the test article are plated with TA100 on selective agar in the presence and absence of metabolic activation. Toxicity is detectable by (1) a decrease in the number of revertant colonies occurring per plate and/or (2) by a thinning or disappearance of the background bacterial lawn. Colonies are machine counted unless otherwise noted.

PRELIMINARY TOXICITY DETERMINATION

OF TEST ARTICLE

T2039-A1
Experiment I.D.

T2039.501
Study Number

Table 2

AT0214
Test Article Identification

Without S-9 Activation

Test Article Concentration µg/Plate	TA100 Revertants/ Plate	TA100 Background Bacterial Lawn*
H_2O 50 µl	89	1
0.0001	78	1
0.001	99	1
0.010	95	1
0.033	83	1
0.10	97	1
0.33	82	1
1.0	97	1
10	106	1
100	100	1
1,000	11	4

*Refer to background bacterial lawn evaluation code key

Date Plated 6/30/83

Dilutions of the test article are plated with TA100 on selective agar in the presence and absence of metabolic activation. Toxicity is detectable by (1) a decrease in the number of revertant colonies occurring per plate and/or (2) by a thinning or disappearance of the background bacterial lawn. Colonies are machine counted unless otherwise noted.

Condition of the Background Bacterial Lawn

The condition of the background bacterial lawn is evaluated for each spontaneous/induced revertant plate, both macroscopically and microscopically by using a dissecting microscope. The evaluation is recorded using the following code:

BACTERIAL BACKGROUND LAWN EVALUATION CODES

Code	Definition	Characteristics
1 or blank	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
5	Absent	Distinguished by a complete lack of any microcolony background lawn.

Evidence of test article precipitate of the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Distinguished by noticeable precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
HP	Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficul

Thus, 3-MP would indicate a plate observed to have a moderately reduced background bacterial lawn with a marked amount of precipitate which required a hand count.

The results of the preliminary toxicity study of MA #T2039 indicate that the appropriate maximum dose level to be tested in the mutagenicity assay would be 600 µg per plate.

The results of the mutagenicity assay are presented in Tables 3 through 14. This data was generated in Experiments T2039-B2 through T2039-B4.

In Experiment T2039-B1, all mutagenicity plates were contaminated by bacteria which made accurate quantitation of histidine revertants impossible.

In Experiment T2039-B2, a small increase in TA98 revertants per plate and a doubling in the number of TA1537 revertants per plate was observed in the presence of rat liver microsomes. In order to confirm these observations, the test article was retested in Experiment T2039-B3 over an extended dose range in the presence of rat liver microsomes on TA98 and TA1537. The results with TA1537 are shown in Table 9. Due to the presence of an intermediate sized TA98 colony type that was present on the TA98 plates (this phenomenon has been observed in ours and other laboratories), it was not possible to accurately quantitate the TA98 revertants per plate. A three-fold increase in TA1537 revertants was observed at the 600 µg per plate dose level.

Although a two-fold and a three-fold increase in TA1537 revertants was observed in Experiments B2 and B3, due to the absence of a clear dose response, these results did not meet the criteria for a positive response as defined by the protocol used for this study.

In an attempt to further clarify the response observed on TA1537, the test article was tested on TA97, a recently developed strain that Ames has recommended to be used in place of TA1537 due to its greater sensitivity to some mutagens. In this experiment, TA98 and TA1537 were also used. The results of these studies are shown in Tables 11 through 14. Increases in revertants per plate were observed at the 500 and 600 µg per plate dose levels for all

three tester strains, TA97, TA98 and TA1537. The responses were less than two-fold on TA97 and TA98 and 2.5-fold on TA1537. Again however, none of the responses met the criteria of a positive response.

SALMONELLA MUTAGENICITY ASSAY

Table 3

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B2 — Experiment Number

Strain: TA98
Date Plated: 7/16/83
Cells Seeded: 1.0 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☒
Machine ☐

	*	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
		Concentration (µg per plate)								
Revertants per plate	2	32	34	37	29	27	37			
	2	27	29	32	38	48	50			
	2	36	30	28	30	32	36			
Averaged Revertants		32	31	32	32	36	41			
Standard Deviation		5	3	5	5	11	8			

T2039-B2 — Experiment Number

Strain: TA98
Date Plated: 7/16/83
Cells Seeded: 1.0 x10^8
Liver Microsomes: None
Colonies Counted by:
Hand ☒
Machine ☐

	*	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
		Concentration (µg per plate)								
Revertants per plate	2	30	20	27	27	28	26			
	2	17	23	20	21	22	24			
	2	18	19	8	16	15	29			
Averaged Revertants		22	21	18	21	22	26			
Standard Deviation		7	2	10	6	7	3			

Form No. MA-160
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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

Table 4

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B2 — Experiment Number

Strain: TA100
Date Plated: 7/16/83
Cells Seeded: 0.8 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☐
Machine ☒

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 6.0	30	150	300	600			
*						2			
Revertants per plate	160	175	164	162	160	154			
	140	163	155	168	204	2 173			
	180	177	162	144	173	2 233			
Averaged Revertants	160	172	160	158	179	187			
Standard Deviation	20	8	5	12	23	41			

T2039-B2 — Experiment Number

Strain: TA100
Date Plated: 7/16/83
Cells Seeded: 0.8 x10^8
Liver Microsomes: None
Colonies Counted by:
Hand ☐
Machine ☒

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 6.0	30	150	300	600			
*						2			
Revertants per plate	129	136	129	136	185	134			
	157	93	118	107	179	2 131			
	122	130	139	135	158	3 102			
Averaged Revertants	136	120	129	126	174	122			
Standard Deviation	19	23	11	16	14	18			

*Background bacterial lawn evaluation code

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SALMONELLA MUTAGENICITY ASSAY

Table 5

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B2 — Experiment Number

Strain: TA1535
Date Plated: 7/16/83
Cells Seeded: 0.7 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
	Concentration (µg per plate)								
*						1			
Revertants per plate	11	16	13	15	12	10			
	16	13	11	14	7	16			
	15	9	13	11	14	7			
Averaged Revertants	14	13	12	13	11	11			
Standard Deviation	3	4	1	2	4	5			

T2039-B2 — Experiment Number

Strain: TA1535
Date Plated: 7/16/83
Cells Seeded: 0.7 x10^8
Liver Microsomes: None
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
	Concentration (µg per plate)								
*						2			
Revertants per plate	16	14	20	31	30	9			
	26	8	20	19	23	2 16			
	23	20	24	16	18	2 15			
Averaged Revertants	22	14	21	22	24	13			
Standard Deviation	5	6	2	8	6	4			

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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

Table 6

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B2 — Experiment Number

Strain: TA1537
Date Plated: 7/16/83
Cells Seeded: 0.8 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 6.0	30	150	300	600			
*						2			
Revertants per plate	8	3	7	8	10	16			
						2			
	4	4	7	4	7	10			
						2			
	7	8	8	8	8	14			
Averaged Revertants	6	5	7	7	8	13			
Standard Deviation	2	3	1	2	2	3			

T2039-B2 — Experiment Number

Strain: TA1537
Date Plated: 7/16/83
Cells Seeded: 0.8 x10^8
Liver Microsomes: None
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	Concentration (µg per plate) 6.0	30	150	300	600			
*						2			
Revertants per plate	8	6	8	2	4	6			
						2			
	7	2	4	3	9	8			
						2			
	2	8	5	7	5	6			
Averaged Revertants	6	5	6	4	6	7			
Standard Deviation	3	3	2	3	3	1			

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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENICITY ASSAY

Table 7

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B2 — Experiment Number

Strain: TA1538
Date Plated: 7/16/83
Cells Seeded: 0.6 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
	Concentration (µg per plate)								
*						2			
Revertants per plate	26	23	24	19	25	16			
	21	21	30	22	24	2 16			
	20	21	19	24	20	2 12			
Averaged Revertants	22	22	24	22	23	15			
Standard Deviation	3	1	6	3	3	2			

T2039-B2 — Experiment Number

Strain: TA1538
Date Plated: 7/16/83
Cells Seeded: 0.6 x10^8
Liver Microsomes: None
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	6.0	30	150	300	600			
	Concentration (µg per plate)								
*						2			
Revertants per plate	11	11	7	10	14	5			
	12	16	13	12	10	2 8			
	13	14	17	13	14	2 10			
Averaged Revertants	12	14	12	12	13	8			
Standard Deviation	1	3	5	2	2	3			

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*Background bacterial lawn evaluation code

SALMONELLA MUTAGENESIS ASSAY

Positive Controls

Table 8

T2039.501	T2039-B2	AT0214
Study Number	Experiment Number	Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
7/16/83	TA98	2-Aminoanthracene	4.0 µg	Rat Liver	3062	2359	2789	2737	354
7/16/83	TA98	2-Nitrofluorene	5.0 µg	None	781	817	870	823	45
7/16/83	TA100	2-Aminoanthracene	4.0 µg	Rat Liver	2371	2341	2292	2335	40
7/16/83	TA100	Sodium Azide	5.0 µg	None	1427	1401	1429	1419	16
7/16/83	TA1535	2-Aminoanthracene	4.0 µg	Rat Liver	212	185	192	196	14
7/16/83	TA1535	Sodium Azide	5.0 µg	None	1233	1306	1306	1282	42
7/16/83	TA1537	2-Aminoanthracene	4.0 µg	Rat Liver	325	253	274	284	37
7/16/83	TA1537	9-Aminoacridine	75 µg	None	404	258	218	293	98
7/16/83	TA1538	2-Aminoanthracene	4.0 µg	Rat Liver	1952	2180	2025	2052	116
7/16/83	TA1538	2-Nitrofluorene	5.0 µg	None	1450	1465	1413	1443	27

Colonies were machine counted.

SALMONELLA MUTAGENICITY ASSAY
Table 9

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B3 — Experiment Number

		Solvent Control H_2O 50 µl	Concentration (µg per plate) 150	300	600	700	800			
Strain: TA1537	*				2	2	3			
Date Plated: 7/20/83	Revertants per plate	3	5	7	12	7	9			
Cells Seeded: 1.1 x10^8					2	2	3			
Liver Microsomes: Rat		5	7	5	12	6	12			
Colonies Counted by:					2	2	3			
Hand ☒		5	9	10	11	5	7			
Machine ☐	Averaged Revertants	4	7	7	12	6	9			
	Standard Deviation	1	2	3	1	1	3			

Experiment Number

		Solvent Control	Concentration (µg per plate)							
Strain:	*									
Date Plated:	Revertants per plate									
Cells Seeded: x10^8										
Liver Microsomes:										
Colonies Counted by:										
Hand ☐										
Machine ☐	Averaged Revertants									
	Standard Deviation									

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

Table 10

T2039.501 Study Number	T2039-B3 Experiment Number	AT0214 Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
7/20/83	TA1537	2-Aminoanthracene	4.0 µg	Rat Liver	292	277	292	287	9

Colonies were machine counted.

SALMONELLA MUTAGENICITY ASSAY

Table 11

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B4 — Experiment Number

		Solvent Control H_2O 50 µl	Concentration (µg per plate) 150	300	500	600	700			
Strain: TA98	*				2	2	3			
Date Plated: 7/23/83	Revertants per plate	28	23	25	35	39	24			
Cells Seeded: 1.1 x10^8					2	2	3			
Liver Microsomes: Rat		29	31	33	38	46	28			
Colonies Counted by:					2	2	3			
Hand ☒		29	28	29	39	35	27			
Machine ☐	Averaged Revertants	29	27	29	37	40	26			
	Standard Deviation	1	4	4	2	6	2			

_____ — Experiment Number

		Solvent Control	Concentration (µg per plate)							
Strain: _____	*									
Date Plated: _____	Revertants per plate									
Cells Seeded: _____ x10^8										
Liver Microsomes: _____										
Colonies Counted by:										
Hand ☐										
Machine ☐	Averaged Revertants									
	Standard Deviation									

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SALMONELLA MUTAGENICITY ASSAY
Table 12

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B4 — Experiment Number

Strain: TA1537
Date Plated: 7/23/83
Cells Seeded: 1.0 x10^8
Liver Microsomes: Rat
Colonies Counted by:
Hand ☒
Machine ☐

	Solvent Control H_2O 50 µl	150	300	500	600	700			
*				2	2	3			
Revertants per plate	3	9	5	9	13	5			
				2	2	3			
	3	7	11	11	8	8			
				2	2	3			
	6	6	6	9	8	3			
Averaged Revertants	4	7	7	10	10	5			
Standard Deviation	2	2	3	1	3	3			

Concentration (µg per plate)

_____ — Experiment Number

Strain: _____
Date Plated: _____
Cells Seeded: _____ x10^8
Liver Microsomes: _____
Colonies Counted by:
Hand ☐
Machine ☐

	Solvent Control								
*									
Revertants per plate									
Averaged Revertants									
Standard Deviation									

Concentration (µg per plate)

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SALMONELLA MUTAGENICITY ASSAY

Table 13

T2039.501 — Study Number

AT0214 — Test Article Identification

T2039-B4 — Experiment Number

		Solvent Control H_2O 50 µl	Concentration (µg per plate) 150	300	500	600	700			
Strain: TA97	*				2	2	3			
Date Plated: 7/23/83	Revertants per plate	84	105	117	144	104	98			
Cells Seeded: 0.5 x10^8					2	2	3			
Liver Microsomes: Rat		96	99	93	125	121	95			
Colonies Counted by:					2	2	3			
Hand ☐		88	101	80	120	114	102			
Machine ☒	Averaged Revertants	89	102	97	130	113	98			
	Standard Deviation	6	3	19	13	9	4			

_____ — Experiment Number

		Solvent Control	Concentration (µg per plate)							
Strain: _____	*									
Date Plated: _____	Revertants per plate									
Cells Seeded: _____ x10^8										
Liver Microsomes: _____										
Colonies Counted by:										
Hand ☐										
Machine ☐	Averaged Revertants									
	Standard Deviation									

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

Table 14

T2039.501	T2039-B4	AT0214
Study Number	Experiment Number	Test Article Identification

Date Plated	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per plate	S.D.
7/23/83	TA98	2-Aminoanthracene	4.0 µg	Rat Liver	2467	2776	2819	2687	192
7/23/83	TA1537	2-Aminoanthracene	4.0 µg	Rat Liver	242	292	305	280	33
7/23/83	TA97	2-Aminoanthracene	3.0 µg	Rat Liver	1320	1298	1508	1375	115

Colonies were machine counted.

APPENDIX

PROTOCOL AMENDMENT

Date: July 28, 1983

Sponsor:

Sponsor's Test Article Designation: AT0214

Study No.: T2039.501

Protocol No.: SPGT501 112382

Protocol Title: Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test)

1. TA97 was used in addition to the five standard tester strains.

Reason for Amendment:

Ames has shown that TA97 is more sensitive to some mutagens than is TA1537.[1] In our attempt to clarify the equivocal response observed on TA1537, the Sponsor agreed to the suggestion of using TA97 in Experiment T2039-B4.

[1]Levin, et al. A new Salmonella tester strain, TA97, for the detection of frameshift mutagens. Mutation Research 94:315-330, 1982.

APPROVAL:

Steve R. Haworth 7/28/83

Steve R. Haworth, Ph.D. Date
Study Director

9/15/83

Ph.D. Date
Study Coordinator

Catalog No. 82-501

SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST)

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article (or its metabolites) based on its ability to induce back mutations at selected loci of several strains of Salmonella typhimurium in the presence and absence of exogenous metabolic activation.

2.0 TEST ARTICLE

2.1 Identification: AT0214

2.2 Analysis:
The sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article (see attached Test Article Characterization form) and the stability of the dosing solutions.

3.0 SPONSOR

3.1 Name:

3.2 Address:

3.3 Authorized Representative:

4.0 TESTING FACILITY

4.1 Name: Division of Genetic Toxicology
Microbiological Associates

4.2 Address: 5221 River Road
Bethesda, Maryland 20816

4.3 Study Location: Rockville Laboratory

4.4 Study Director: Steve R. Haworth, Ph.D.

5.0 TEST SYSTEM

The Ames Test has been shown to be a sensitive, rapid, accurate indicator of the mutagenic activity of a wide range of chemical classes.

The tester strains to be used will be the Salmonella typhimurium histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538 as described by Ames (Ames, et al., Mutation Research 31:347-364, 1975).

GENOTYPE OF THE TA STRAINS USED FOR MUTAGEN TESTING

Histidine mutation			Additional mutations		
hisG46	hisC3076	hisD3052	LPS	Repair	R factor
TA1535	TA1537	TA1538	rfa	uvrB	-
TA100		TA98	rfa	uvrB	+R

All of the tester strains contain, in addition to a mutation in the histidine operon, two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa mutation causes a loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide layer of the cell wall. The resulting cell wall deficiency increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded by a normal intact cell wall.

The second mutation is a deletion in the uvrB gene which results in a deficient DNA excision-repair system. This deficiency results in greatly enhanced sensitivity to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Finally, strains TA98 and TA100 also contain the pkM101 plasmid (carrying the R-factor) which further increases the sensitivity of these two strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be due to its coding for an error-prone DNA repair polymerase.

TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy)

by frame shift mutagens. TA100 and TA1535 are reverted by mutagens that cause base substitutions.

5.1 Source
Tester strains in use at Microbiological Associates were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

5.2 Storage
All Frozen Permanent and Working Stocks of the tester strains will be stored in liquid nitrogen. Working Stocks will be prepared by growing a fresh overnight culture inoculated by a scrape of the Frozen Permanent Stock, adding DMSO (.09 ml/ml of culture) and freezing away small aliquots (0.1 - 0.2 ml) in glass vials.

5.3 Overnight Culture Preparation
Overnight cultures will be prepared by removing a Working Stock vial from the liquid nitrogen freezer and allowing it to thaw. A loopful of the thawed aliquot will be transferred to a baffled shake flask containing approximately 50 ml of culture media. The inoculated flask will be placed in a shaker/incubator at 37°C.

5.4 Harvesting of Cultures
All cultures will be harvested by monitoring optical density rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately $1\text{-}2 \times 10^9$ cells per ml.

5.5 Genotype Characterization
On the day of their use in the mutagenicity assay, all tester strain cultures will be checked for the following genetic markers:

5.5.1 The presence of the rfa wall mutation will be confirmed by demonstration of sensitivity to crystal violet.

5.5.2 The presence of the pkM101 plasmid will be confirmed for tester strains TA98 and TA100 by demonstration of resistance to Ampicillin.

5.5.3 Spontaneous reversion frequencies that are characteristic of the respective strains will be demonstrated by plating aliquots of the culture on selective media.

6.0 EXPERIMENTAL DESIGN

The test article will be tested at a minimum of five dose levels along with appropriate solvent and positive controls on tester strains TA98, TA100, TA1535, TA1537 and TA1538 with and without metabolic activation. Following an approximate 48 hour incubation at 37°C, revertant colonies per plate will be counted.

6.1 Dose Levels

Using TA100 as the indicator strain, each test article will be checked for toxicity up to a concentration of 10 mg/plate if solubility/miscibility permits. Test articles which exhibit limited solubility/miscibility will be tested for toxicity up to the maximum workable concentration attainable in the solvent of choice. The toxicity determination will be conducted both in the presence and absence of exogenous metabolic activation. An aliquot from each of at least eight dilutions of the test article will be plated with an overnight TA100 culture on selective minimal agar. Toxicity is detectable as a decrease in the number of revertant colonies occurring per plate and/or by a thinning or disappearance of the background bacterial lawn. The highest concentration of test article used in the subsequent mutagenicity assay will be that which gives a detectable reduction of revertants on the selective plates and/or a thinning or disappearance of the background bacterial lawn.

If no toxicity is observed, then the highest dose level used in the mutagenicity assay will be 10 mg/plate unless:

1) The test article exhibits limited solubility or is not uniformly dispersible in the solvent of choice.

2) The test article precipitates heavily in the top agar.

3) There is insufficient test article available to either demonstrate toxicity or achieve a maximum dose level of 10 mg/plate.

4) The study coordinator indicates an alternate top dose level.

6.2 Frequency and Route of Administration

The test system will be exposed to the test article via the plate incorporation methodology originally described by Ames (Ames, et al., Mutation Research 31:347-364, 1975). This methodology has been

shown to detect a wide range of classes of chemical mutagens. All dose levels of test article, solvent controls and positive controls will be plated in triplicate.

6.3 Exogenous Metabolic Activation

6.3.1 Liver Microsomal Enzymes - S-9 Homogenate

6.3.1.1 Homogenate Preparation
The preparation of the microsomal enzyme fraction will be carried out with sterile glassware and solutions at 0-4°C. Excised livers will be placed in approximately 20 ml of 0.15M KCl contained in a pre-weighed beaker. After the liver is weighed, it will be transferred to another beaker containing 3 volumes of 0.15M KCl (3 ml/g of wet liver) where it will be minced with sterile scissors. The minced liver will be homogenized and centrifuged at 9000 x g for 10 minutes. The supernatant (referred to by Ames as the S-9 fraction) will be decanted, and small aliquots will be distributed into freezing ampules which will be stored at $\leq$ -70°C.

6.3.1.2 S-9 Characterization
Each batch of S-9 homogenate will be characterized for its ability to metabolize the promutagens 7,12-dimethylbenzanthracene, and 2-aminoanthracene to mutagens as described by deSerres (deSerres, et al., Science 203:563-565, 1979).

6.3.1.3 Species, Strain, Sex, Inducer
Liver microsomal enzymes will be prepared from male Sprague-Dawley rats that have been injected with Aroclor 1254 at 500 mg/kg. The Aroclor will be diluted in corn oil to a concentration of 200 mg/ml. Five days after i.p. injection with the Aroclor, the rats will be sacrificed by decapitation, and their livers will be excised. The rats will be fasted for 12 hours immediately preceding sacrifice.

6.3.2 S-9 Mix

The S-9 mix will be prepared immediately prior to its use in any experimental procedure.

One ml of the microsomal enzyme reaction mixture (S-9 mix) which is added to the soft agar overlay will contain the following components:

H_2O	0.56 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
0.20M $MgCl_2$/0.825M KCl	0.04 ml
0.04M G-6-P	0.10 ml
0.04M NADP	0.10 ml
S-9	0.10 ml
	1.00 ml

Each plate will receive 0.5 ml of the S-9 mix.

6.4 Controls

6.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the assay are listed below:

Strain	Activation	Positive Controls	Conc. per Plate
TA98	+	2-aminoanthracene	4.0 ug
TA98	-	2-nitrofluorene	5.0 ug
TA100	+	2-aminoanthracene	4.0 ug
TA100	-	sodium azide	5.0 ug
TA1535	+	2-aminoanthracene	4.0 ug
TA1535	-	sodium azide	5.0 ug
TA1537	+	2-aminoanthracene	4.0 ug
TA1537	-	9-aminoacridine	75 ug
TA1538	+	2-aminoanthracene	4.0 ug
TA1538	-	2-nitrofluorene	5.0 ug

6.4.2 Solvent Controls

Appropriate solvent controls will be plated for all strains with and without metabolic activation. Solvents compatible with this test system in order of preference include but will not be limited to: Deionized distilled H_2O, dimethylsulfoxide (CAS #67-68-5),

acetone (CAS #67-64-1), and ethanol (CAS #64-17-5).

6.4.3 Sterility Controls

6.4.3.1 The most concentrated test article dilution will be checked for sterility.

6.4.3.2 The S-9 mix will be checked for sterility.

6.4.4 Tester Strain Titers
Each tester strain titer will be determined by plating an appropriate dilution of each overnight culture on complete agar.

7.0 METHODS

7.1 Plating Procedures for the Mutagenicity Assay
The test article will be solubilized and serially diluted immediately before its use in the mutagenicity assay. S-9 mix will also be prepared immediately prior to its use in the mutagenicity assay.

At least five doses of the test article will be plated with the appropriate tester strains, both with and without metabolic activation.

Without metabolic activation, 50 ul of tester strain and 50 ul of solvent or test article will be added to 2.5 ml of molten selective top agar at 45°C. With metabolic activation, 50 ul of tester strain, 50 ul of solvent or test article solution and 0.5 ml of S-9 mix will be added to 2.0 ml of molten selective top agar at 45°C. After vortexing, the mixture will be overlayed onto the surface of 25 ml of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 hours at 37°C. When necessary, aliquots of other than 50 ul of test article/solvent will be plated.

7.2 Test System Identification
Each plate will be labeled using indelible ink with a code system which identifies the test article, test phase, dose level, strain and activation type as described in detail in Microbiological Associates' Microbial Mutagenesis Standard Operating Procedures.

7.3 Colony Counting
Revertant colonies for a given tester strain within a given test article dilution series will be counted

either entirely by automated colony counter or entirely by hand. Plates with sufficient test article precipitate to interfere with automated colony counting will be counted manually.

7.3.1 Background Bacterial Lawn Evaluation
The condition of the background bacterial lawn on plates in the assay will be evaluated for evidence of test article toxicity and precipitate. Evidence of toxicity will be scored relative to the solvent control plate and recorded along with the revertant count for that plate.

7.4 Analysis of Data
For all replicate platings, the mean revertants per plate and the standard deviation will be calculated.

8.0 EVALUATION OF TEST RESULTS

For a test article to be considered positive, it must cause at least a doubling in the mean revertants per plate of at least one tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article. In those cases where the observed dose-responsive increase in TA1537 or TA1538 revertants per plate is less than three-fold, the response must be reproducible.

9.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the assay to be considered valid:

9.1 Tester Strain Integrity

9.1.1 rfa Wall Mutation
In order to demonstrate the presence of the deep rough wall mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

9.1.2 pkM101 Plasmid R-factor
In order to demonstrate the presence of the pkM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to Ampicillin.

9.1.3 Characteristic Number of Spontaneous Revertants
All tester strain cultures must exhibit a characteristic number of spontaneous revertants

per plate. The acceptable ranges are as follows:

TA98	10 - 50
TA100	80 - 240
TA1535	5 - 45
TA1537	3 - 21
TA1538	5 - 35

9.1.4 Tester Strain Titers
In order to ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than $1x10^9$ but less than $4x10^9$.

9.1.5 Positive Control Values
Positive control values must exhibit at least a three fold increase in the number of revertants per plate over the average value for the solvent control for the respective strain.

9.2 Toxicity

9.2.1 Minimum Number of Dose Levels
A minimum of three non-toxic dose levels are required to evaluate assay data.

10.0 FINAL REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of data.

Results of the preliminary toxicity determinations will be presented which will include the number of revertants per plate and a background bacterial lawn evaluation for each dose level.

Results presented for the mutagenicity assay will include the number of revertants per plate with a corresponding background bacterial lawn evaluation, along with a mean and standard deviation for all replicate platings.

11.0 RECORD AND TEST ARTICLE ARCHIVES

11.1 Records
Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates in accordance with the Terms and Conditions.

11.2 Test Article
A sample of the Test Article will be held in storage in accordance with the Terms and Conditions.

12.0 GOOD LABORATORY PRACTICES

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? No
If so, to which agency or agencies? _____

Does the sponsor request that samples of the Test Article dosing solutions be returned? No

13.0 SCHEDULE OF EVENTS

13.1 Proposed Initiation Date: ~~6/29/83~~ SRH 7/28/83 6/30/83

13.2 Scheduled Completion Date: 7/29/83

14.0 REFERENCES

Ames, B.N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/Mammalian-Microsome Mutagenicity test. Mutation Research 31:347-364, 1975.

deSerres, et al., The Salmonella Mutagenicity Assay: Recommendations, Science 203:563-565, 1979.

SPONSOR'S AUTHORIZED REPRESENTATIVE

6/1/83
DATE PROTOCOL APPROVED BY SPONSOR

Steve R. Haworth 6/15/83
STUDY DIRECTOR DATE

MICROBIOLOGICAL ASSOCIATES